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(21) International Application Number: PCT/US98/13966 (22) International Filing Date: 6 July 1998 (06.07.98) (30) Priority Data: 08/889,296 8 July 1997 (08.07.97) US (63) Related by Continuation (CON) or Continuation-in-Part (CIP) to Earlier Application US 08/889,296 (CON) Filed on 8 July 1997 (08.07.97) (71) Applicant (for all designated States except US): ISIS PHARMACEUTICALS, INC. [US/US]; Carlsbad Research Center, 2292 Faraday Avenue, Carlsbad, CA 92008 (US). (72) Inventors; and (75) Inventors/Applicants (for US only): MONIA, Brett, P. [US/US]; 7605 Nueva Castilla Way, La Costa, CA 92009 (US). COWSERT, Lex, M. [US/US]; 3008 Newshire Street, Carlsbad, CA 92008 (US). MANOHARAN, Muthiah [US/US]; 7634 Reposado Drive, Carlsbad, CA 92009 (US). (74) Agents: LICATA, Jane, Massey et al.; Law Offices of Jane Massey Licata, 66 E. Main Street, Marlton, NJ 08053 (US).		(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, GW, HR, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG). Published <i>With international search report.</i>	
(54) Title: ANTISENSE OLIGONUCLEOTIDE INHIBITION OF RAS			
(57) Abstract Compositions and methods are provided for the modulation of ras expression. Oligonucleotides are provided which are targeted to nucleic acids encoding human ras. Oligonucleotides specifically hybridizable with mRNA encoding human H-ras, Ki-ras and N-ras are provided. Such oligonucleotides can be used for therapeutics and diagnostics as well as for research purposes. Methods are also disclosed for modulating ras gene expression in cells and tissues using the oligonucleotides provided, and for specific modulation of expression of activated ras. Methods for diagnosis, detection and treatment of conditions associated with ras are also disclosed.			

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ANTISENSE OLIGONUCLEOTIDE INHIBITION OF RAS

This application is a continuation-in-part of U.S. Patent Application No. 08/411,734, filed April 3, 1995 which is a U.S. national phase application of PCT/US93/09346, filed
5 October 1, 1993, which is a continuation-in-part and foreign filing of U.S. Patent Application No. 958,134, filed October 5, 1992, and U.S. Patent Application No. 08/007,996, filed January 21, 1993, all of which are assigned to the assignee of the present invention and are incorporated herein by
10 reference in their entirety.

FIELD OF THE INVENTION

This invention relates to compositions and methods for the inhibition of expression of ras, a naturally occurring protein which occasionally converts to an activated form that
15 has been implicated in tumor formation. Antisense oligonucleotides targeted to H-, Ki- and N-ras are provided. This invention is further directed to the detection of both normal and activated forms of the ras gene in cells and tissues, and can form the basis for research reagents and kits
20 both for research and diagnosis. Furthermore, this invention is directed to prevention and treatment of conditions associated with ras.

BACKGROUND OF THE INVENTION

Alterations in the cellular genes which directly or indirectly control cell growth and differentiation are considered to be the main cause of cancer. There are some
5 thirty families of genes, called oncogenes, which are implicated in human tumor formation. Members of one such family, the ras gene family, are frequently found to be mutated in human tumors. In their normal state, proteins produced by the ras genes are thought to be involved in normal
10 cell growth and maturation. Mutation of the ras gene, causing an amino acid alteration at one of three critical positions in the protein product, results in conversion to a form which is implicated in tumor formation. A gene having such a mutation is said to be "mutant" or "activated." Unmutated ras
15 is called "wild-type" or "normal" ras. It is thought that such a point mutation leading to ras activation can be induced by carcinogens or other environmental factors. Over 90% of pancreatic adenocarcinomas, about 50% of adenomas and adenocarcinomas of the colon, about 50% of adenocarcinomas of
20 the lung and carcinomas of the thyroid, and a large fraction of malignancies of the blood such as acute myeloid leukemia and myelodysplastic syndrome have been found to contain activated ras oncogenes. Overall, some 10 to 20% of human tumors have a mutation in one of the three ras genes (H-ras,
25 Ki-ras, or N-ras).

It is presently believed that inhibiting expression of activated oncogenes in a particular tumor cell might force the cell back into more normal growth. For example, Feramisco et al., *Nature* 1985, 314, 639-642, demonstrated that if cells
30 transformed to a malignant state with an activated ras gene are microinjected with antibody which binds to the protein product of the ras gene, the cells slow their rate of proliferation and adopt a more normal appearance. This has been interpreted as support for the involvement of the product
35 of the activated ras gene in the uncontrolled growth typical

of cancer cells.

There is a great desire to provide compositions of matter which can modulate the expression of ras, and particularly to provide compositions of matter which specifically modulate the expression of activated ras. It is greatly desired to provide methods of diagnosis and detection of nucleic acids encoding ras in animals. It is also desired to provide methods of diagnosis and treatment of conditions arising from ras activation. In addition, improved research kits and reagents for detection and study of nucleic acids encoding ras are desired.

Inhibition of oncogene expression has been accomplished using retroviral vectors or plasmid vectors which express a 2-kilobase segment of the Ki-ras protooncogene RNA in antisense orientation. Mukhopadhyay, T. et al. (1991) *Cancer Research* 51, 1744-1748; PCT Patent Application PCT/US92/01852 (WO 92/15680); Georges, R.N. et al. (1993) *Cancer Research*, 53, 1743-1746.

Antisense oligonucleotide inhibition of oncogenes has proven to be a useful tool in understanding the roles of various oncogene families. Antisense oligonucleotides are small oligonucleotides which are complementary to the "sense" or coding strand of a given gene, and as a result are also complementary to, and thus able to stably and specifically hybridize with, the mRNA transcript of the gene. Holt et al., *Mol. Cell Biol.* 1988, 8, 963-973, have shown that antisense oligonucleotides hybridizing specifically with mRNA transcripts of the oncogene c-myc, when added to cultured HL60 leukemic cells, inhibit proliferation and induce differentiation. Anfossi et al., *Proc. Natl. Acad. Sci.* 1989, 86, 3379-3383, have shown that antisense oligonucleotides specifically hybridizing with mRNA transcripts of the c-myc oncogene inhibit proliferation of human myeloid leukemia cell lines. Wickstrom et al., *Proc. Nat. Acad. Sci.* 1988, 85, 1028-1032, have shown that expression of the protein product

of the c-myc oncogene as well as proliferation of HL60 cultured leukemic cells are inhibited by antisense oligonucleotides hybridizing specifically with c-myc mRNA. United States Patent No: 4,871,838 (Bos et al.) discloses
5 oligonucleotides complementary to a mutation in codon 13 of N-ras to detect said mutation. United States Patent No: 4,871,838 (Bos et al.) discloses molecules useful as probes for detecting a mutation in DNA which encodes a ras protein.

In all these cases, instability of unmodified
10 oligonucleotides has been a major problem, as they are subject to degradation by cellular enzymes. PCT/US88/01024 (Zon et al.) discloses phosphorothioate oligonucleotides hybridizable to the translation initiation region of the amplified c-myc oncogene to inhibit HL-60 leukemia cell growth and DNA
15 synthesis in these cells. Tidd et al., *Anti-Cancer Drug Design* 1988, 3, 117-127, evaluated methylphosphonate antisense oligonucleotides hybridizing specifically to the activated N-ras oncogene and found that while they were resistant to biochemical degradation and were nontoxic in cultured human
20 HT29 cells, they did not inhibit N-ras gene expression and had no effect on these cells. Chang et al. showed that both methylphosphonate and phosphorothioate oligonucleotides hybridizing specifically to mRNA transcripts of the mouse Balb-ras gene could inhibit translation of the protein product
25 of this gene *in vitro*. Chang et al., *Anti-Cancer Drug Design* 1989, 4, 221-232; Brown et al., *Oncogene Research* 1989, 4, 243-252. It was noted that T_m was not well correlated with antisense activity of these oligonucleotides against *in vitro* translation of the ras p21 protein product. Because the
30 antisense oligonucleotides used by Chang et al. hybridize specifically with the translation initiation region of the ras gene, they are not expected to show any selectivity for activated ras and the binding ability of these oligonucleotides to normal (wild-type) vs. mutated (activated)
35 ras genes was not compared.

Helene and co-workers have demonstrated selective inhibition of activated (codon 12 G-T transition) H-ras mRNA expression using a 9-mer phosphodiester linked to an acridine intercalating agent and/or a hydrophobic tail. This compound displayed selective targeting of mutant ras message in both Rnase H and cell proliferation assays at low micromolar concentrations. Saison-Behmoaras, T. et al., *EMBO J.* 1991, 10, 1111-1118. Chang and co-workers disclose selective targeting of mutant H-ras message; this time the target was H-ras codon 61 containing an A-T transversion and the oligonucleotide employed was either an 11-mer methylphosphonate or its psoralen derivative. These compounds, which required concentrations of 7.5-150 μ M for activity, were shown by immunoprecipitation to selectively inhibit mutant H-ras p21 expression relative to normal p21. Chang et al., *Biochemistry* 1991, 30, 8283-8286.

SUMMARY OF THE INVENTION

The present invention relates to antisense oligonucleotides which are targeted to human ras, and methods of using them. More specifically, the present invention provides oligonucleotides which are targeted to mRNA encoding human H-ras, Ki-ras and N-ras and which are capable of inhibiting ras expression. Oligonucleotides targeted to a 5' untranslated region, translation initiation site, coding region or 3' untranslated region of human N-ras are provided. Methods of modulating ras expression, of inhibiting the proliferation of cancer cells and of treating conditions associated with ras are provided. These methods employ the oligonucleotides of the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a series of 8 panels showing inhibition of ras in a dose-dependent manner. Solid lines are activity against wild-type (normal) ras, dotted lines show activity

against activated (mutant) ras.

Figure 2 is a bar graph showing antisense activities of a uniform deoxy phosphorothioate and shortened chimeric oligonucleotides against ras-luciferase.

5 Figure 3 is a line graph showing correlation between antisense activity and ability to activate RNase H as a function of deoxy gap length using phosphorothioate 2'-O-methyl oligonucleotides targeted against ras.

Figure 4 is a line graph showing anti-tumor activity of
10 ISIS 2503 against A549 human cell tumors in nude mice.

Figure 5 is a line graph showing anti-tumor activity of ras oligo ISIS 2503, administered with cationic lipid, against A549 human cell tumors in nude mice.

Figure 6 is a bar graph showing antisense inhibition of
15 Ki-ras mRNA expression in three human colon carcinoma cell lines, Calu1, SW480 and SW620.

Figure 7 is a bar graph showing inhibition of SW480 human carcinoma cell line proliferation by Ki-ras specific oligonucleotides ISIS 6957 and ISIS 6958.

20 Figure 8 is a bar graph showing reduction of H-ras mRNA levels by 2'-MOE analogs of ISIS 2503 (SEQ ID NO: 2). Black bars: 150 nM oligonucleotide dose; Diagonal hatched bars: 50 nM dose; horizontal hatched bars: 15 nM dose.

Figure 9 is a bar graph showing reduction of H-ras mRNA
25 levels by MMI analogs of ISIS 2503 (SEQ ID NO: 2). Black bars: 500 nM oligonucleotide dose; Diagonal hatched bars: 100 nM dose; horizontal hatched bars: 50 nM dose.

Figure 10 is a bar graph showing reduction of N-ras mRNA levels by oligonucleotides 14686-14694, 14677 and 14678. Black
30 bars: 400 nM oligonucleotide dose; Diagonal hatched bars: 200 nM dose; horizontal hatched bars: 100 nM dose.

DETAILED DESCRIPTION OF THE INVENTION

Malignant tumors develop through a series of stepwise, progressive changes that lead to the loss of growth control
35 characteristic of cancer cells, i.e., continuous unregulated

proliferation, the ability to invade surrounding tissues, and the ability to metastasize to different organ sites. Carefully controlled *in vitro* studies have helped define the factors that characterize the growth of normal and neoplastic cells and have led to the identification of specific proteins that control cell growth and differentiation. In addition, the ability to study cell transformation in carefully controlled, quantitative *in vitro* assays has led to the identification of specific genes capable of inducing the transformed cell phenotype. Such cancer-causing genes, or oncogenes, are believed to acquire transformation-inducing properties through mutations leading to changes in the regulation of expression of their protein products. In some cases such changes occur in non-coding DNA regulatory domains, such as promoters and enhancers, leading to alterations in the transcriptional activity of oncogenes, resulting in over- or under-expression of their gene products. In other cases, gene mutations occur within the coding regions of oncogenes, leading to the production of altered gene products that are inactive, overactive, or exhibit an activity that is different from the normal (wild-type) gene product.

To date, more than 30 cellular oncogene families have been identified. These genes can be categorized on the basis of both their subcellular location and the putative mechanism of action of their protein products. The ras oncogenes are members of a gene family which encode related proteins that are localized to the inner face of the plasma membrane. ras proteins have been shown to be highly conserved at the amino acid level, to bind GTP with high affinity and specificity, and to possess GTPase activity. Although the cellular function of ras gene products is unknown, their biochemical properties, along with their significant sequence homology with a class of signal-transducing proteins known as GTP binding proteins, or G proteins, suggest that ras gene products play a fundamental role in basic cellular regulatory

functions relating to the transduction of extracellular signals across plasma membranes.

Three ras genes, designated H-ras, Ki-ras, and N-ras, have been identified in the mammalian genome. Mammalian ras
5 genes acquire transformation-inducing properties by single point mutations within their coding sequences. Mutations in naturally occurring ras oncogenes have been localized to codons 12, 13, and 61. The sequences of H-ras, Ki-ras and N-ras are known. Capon et al., *Nature* 302 1983, 33-37; Kahn
10 et al., *Anticancer Res.* 1987, 7, 639-652; Hall and Brown, *Nucleic Acids Res.* 1985, 13, 5255-5268. The most commonly detected activating ras mutation found in human tumors is in codon 12 of the H-ras gene in which a base change from GGC to GTC results in a glycine-to-valine substitution in the GTPase
15 regulatory domain of the ras protein product. Tabin, C.J. et al., *Nature* 1982, 300, 143-149; Reddy, P.E. et al., *Nature* 1982, 300, 149-152; Taparowsky, E. et al., *Nature* 1982, 300, 762-765. This single amino acid change is thought to abolish normal control of ras protein function, thereby converting a
20 normally regulated cell protein to one that is continuously active. It is believed that such deregulation of normal ras protein function is responsible for the transformation from normal to malignant growth. It is therefore believed that inhibition of ras expression is useful in treatment and/or
25 prevention of malignant conditions, i.e., cancer and other hyperproliferative conditions.

The H-ras gene has recently been implicated in a serious cardiac arrhythmia called long Q-T syndrome, a hereditary condition which often causes sudden death if treatment is not
30 given immediately. Frequently, there are no symptoms prior to the onset of the erratic heartbeat. Whether the H-ras gene is precisely responsible for long Q-T syndrome is unclear. However, there is an extremely high correlation between inheritance of this syndrome and the presence of a particular

variant of the chromosome 11 region surrounding the H-ras gene. Therefore, the H-ras gene is a useful indicator of increased risk of sudden cardiac death due to the long Q-T syndrome.

5 N-ras was first identified as an oncogene in gene transfer experiments. Hall et al. *Nature* 1983, 303: 396-400. Its activation was characterized by Taparowsky et al. *Cell* 1983 34: 581-6. Activated N-ras is found in many hematologic neoplasms and solid tumors, suggesting a role for N-ras in the
10 development or maintenance of hyperproliferative conditions.

The present invention provides oligonucleotides for inhibition of human ras gene expression. Such oligonucleotides specifically hybridize with selected DNA or mRNA deriving from a human ras gene. The invention also
15 provides oligonucleotides for selective inhibition of expression of the mutant form of ras. This relationship between an oligonucleotide and its complementary nucleic acid target to which it hybridizes is commonly referred to as "antisense". "Targeting" an oligonucleotide to a chosen
20 nucleic acid target, in the context of this invention, is a multistep process. The process usually begins with identifying a nucleic acid sequence whose function is to be modulated. This may be, for example, a cellular gene (or mRNA made from the gene) whose expression is associated with a
25 particular disease state, or a foreign nucleic acid from an infectious agent. In the present invention, the target is a nucleic acid encoding ras; in other words, the ras gene or mRNA expressed from the ras gene. The targeting process also includes determination of a site or sites within the nucleic
30 acid sequence for the oligonucleotide interaction to occur such that the desired effect- modulation of gene expression- will result. Once the target site or sites have been identified, oligonucleotides are chosen which are sufficiently complementary to the target, i.e., hybridize sufficiently well

and with sufficient specificity, to give the desired modulation.

In the context of this invention "modulation" means either inhibition or stimulation. Inhibition of ras gene
5 expression is presently the preferred form of modulation. This modulation can be measured in ways which are routine in the art, for example by Northern blot assay of mRNA expression or Western blot assay of protein expression as taught in the examples of the instant application. Effects on cell
10 proliferation or tumor cell growth can also be measured, as taught in the examples of the instant application. "Hybridization", in the context of this invention, means hydrogen bonding, also known as Watson-Crick base pairing, between complementary bases, usually on opposite nucleic acid
15 strands or two regions of a nucleic acid strand. Guanine and cytosine are examples of complementary bases which are known to form three hydrogen bonds between them. Adenine and thymine are examples of complementary bases which form two hydrogen bonds between them. "Specifically hybridizable" and
20 "complementary" are terms which are used to indicate a sufficient degree of complementarity such that stable and specific binding occurs between the DNA or RNA target and the oligonucleotide. It is understood that an oligonucleotide need not be 100% complementary to its target nucleic acid
25 sequence to be specifically hybridizable. An oligonucleotide is specifically hybridizable when binding of the oligonucleotide to the target interferes with the normal function of the target molecule to cause a loss of utility, and there is a sufficient degree of complementarity to avoid
30 non-specific binding of the oligonucleotide to non-target sequences under conditions in which specific binding is desired, i.e., under physiological conditions in the case of *in vivo* assays or therapeutic treatment or, in the case of *in vitro* assays, under conditions in which the assays are
35 conducted.

In preferred embodiments of this invention, oligonucleotides are provided which are targeted to mRNA encoding H-ras, Ki-ras or N-ras. In accordance with this invention, persons of ordinary skill in the art will understand that mRNA includes not only the coding region which carries the information to encode a protein using the three letter genetic code, including the translation start and stop codons, but also associated ribonucleotides which form a region known to such persons as the 5'-untranslated region, the 3'-untranslated region, the 5' cap region, intron regions and intron/exon or splice junction ribonucleotides. Thus, oligonucleotides may be formulated in accordance with this invention which are targeted wholly or in part to these associated ribonucleotides as well as to the coding ribonucleotides. In preferred embodiments, the oligonucleotide is targeted to a translation initiation site (AUG codon) or sequences in the coding region, 5' untranslated region or 3'-untranslated region of the ras mRNA. The functions of messenger RNA to be interfered with include all vital functions such as translocation of the RNA to the site for protein translation, actual translation of protein from the RNA, splicing or maturation of the RNA and possibly even independent catalytic activity which may be engaged in by the RNA. The overall effect of such interference with the RNA function is to cause interference with ras protein expression.

The present invention provides oligonucleotides for modulation of ras gene expression. Such oligonucleotides are targeted to nucleic acids encoding ras. As hereinbefore defined, "modulation" means either inhibition or stimulation. Inhibition of ras gene expression is presently the preferred form of modulation.

In the context of this invention, the term "oligonucleotide" refers to an oligomer or polymer of nucleotide or nucleoside monomers consisting of naturally occurring bases, sugars and intersugar (backbone) linkages.

The term "oligonucleotide" also includes oligomers comprising non-naturally occurring monomers, or portions thereof, which function similarly. Such modified or substituted oligonucleotides are often preferred over native forms because of properties such as, for example, enhanced cellular uptake and increased stability in the presence of nucleases.

Certain preferred oligonucleotides of this invention are chimeric oligonucleotides. "Chimeric oligonucleotides" or "chimeras", in the context of this invention, are oligonucleotides which contain two or more chemically distinct regions, each made up of at least one nucleotide. These oligonucleotides typically contain at least one region of modified nucleotides that confers one or more beneficial properties (such as, for example, increased nuclease resistance, increased uptake into cells, increased binding affinity for the RNA target) and a region that is a substrate for enzymes capable of cleaving RNA:DNA or RNA:RNA hybrids. By way of example, RNase H is a cellular endonuclease which cleaves the RNA strand of an RNA:DNA duplex. Activation of RNase H, therefore, results in cleavage of the RNA target, thereby greatly enhancing the efficiency of antisense inhibition of gene expression. Consequently, comparable results can often be obtained with shorter oligonucleotides when chimeric oligos are used, compared to phosphorothioate deoxyoligonucleotides hybridizing to the same target region. Cleavage of the RNA target can be routinely detected by gel electrophoresis and, if necessary, associated nucleic acid hybridization techniques known in the art. In one preferred embodiment, a chimeric oligonucleotide comprises at least one region modified to increase target binding affinity, and, usually, a region that acts as a substrate for RNase H. Affinity of an oligonucleotide for its target (in this case, a nucleic acid encoding ras) is routinely determined by measuring the T_m of an oligonucleotide/target pair, which is the temperature at which the oligonucleotide and target

dissociate; dissociation is detected spectrophotometrically. The higher the T_m , the greater the affinity of the oligonucleotide for the target. In a more preferred embodiment, the region of the oligonucleotide which is
5 modified to increase ras mRNA binding affinity comprises at least one nucleotide modified at the 2' position of the sugar, most preferably a 2'-O-alkyl, 2'-O-alkyl-O-alkyl or 2'-fluoro-modified nucleotide. Such modifications are routinely incorporated into oligonucleotides and these oligonucleotides
10 have been shown to have a higher T_m (i.e., higher target binding affinity) than 2'-deoxyoligonucleotides against a given target. The effect of such increased affinity is to greatly enhance antisense oligonucleotide inhibition of ras gene expression. RNase H is a cellular endonuclease that
15 cleaves the RNA strand of RNA:DNA duplexes; activation of this enzyme therefore results in cleavage of the RNA target, and thus can greatly enhance the efficiency of antisense inhibition. Cleavage of the RNA target can be routinely demonstrated by gel electrophoresis. In another preferred
20 embodiment, the chimeric oligonucleotide is also modified to enhance nuclease resistance. Cells contain a variety of exo- and endo-nucleases which can degrade nucleic acids. A number of nucleotide and nucleoside modifications have been shown to make the oligonucleotide into which they are incorporated more
25 resistant to nuclease digestion than the native oligodeoxynucleotide. Nuclease resistance is routinely measured by incubating oligonucleotides with cellular extracts or isolated nuclease solutions and measuring the extent of intact oligonucleotide remaining over time, usually by gel
30 electrophoresis. Oligonucleotides which have been modified to enhance their nuclease resistance survive intact for a longer time than unmodified oligonucleotides. A variety of oligonucleotide modifications have been demonstrated to enhance or confer nuclease resistance. Oligonucleotides which
35 contain at least one phosphorothioate modification are

presently more preferred. In some cases, oligonucleotide modifications which enhance target binding affinity are also, independently, able to enhance nuclease resistance. A discussion of antisense oligonucleotides and some desirable
5 modifications can be found in De Mesmaeker et al. *Acc. Chem. Res.* 1995, 28:366-374.

Specific examples of some preferred oligonucleotides envisioned for this invention include those containing modified backbones, for example, phosphorothioates,
10 phosphotriesters, methyl phosphonates, short chain alkyl or cycloalkyl intersugar linkages or short chain heteroatomic or heterocyclic intersugar linkages. Most preferred are oligonucleotides with phosphorothioate backbones and those with heteroatom backbones, particularly $\text{CH}_2\text{-NH-O-CH}_2$, $\text{CH}_2\text{-N(CH}_3\text{)-O-CH}_2$ [known as a methylene(methylimino) or MMI
15 backbone], $\text{CH}_2\text{-O-N(CH}_3\text{)-CH}_2$, $\text{CH}_2\text{-N(CH}_3\text{)-N(CH}_3\text{)-CH}_2$ and $\text{O-N(CH}_3\text{)-CH}_2\text{-CH}_2$ backbones, wherein the native phosphodiester backbone is represented as O-P-O-CH_2). The amide backbones disclosed by De Mesmaeker et al. *Acc. Chem. Res.* 1995, 28:366-374) are
20 also preferred. Also preferred are oligonucleotides having morpholino backbone structures (Summerton and Weller, U.S. Patent 5,034,506). In other preferred embodiments, such as the peptide nucleic acid (PNA) backbone, the phosphodiester backbone of the oligonucleotide is replaced with a polyamide
25 backbone, the nucleobases being bound directly or indirectly to the aza nitrogen atoms of the polyamide backbone (Nielsen et al. *Science* 1991, 254, 1497). Oligonucleotides may also contain one or more substituted sugar moieties. Preferred oligonucleotides comprise one of the following at the 2'
30 position: OH, SH, SCH_3 , F, OCN, OCH_2OCH_2 , $\text{OCH}_2\text{O(CH}_2\text{)}_n\text{CH}_3$, $\text{O(CH}_2\text{)}_n\text{NH}_2$ or $\text{O(CH}_2\text{)}_n\text{CH}_3$ where n is from 1 to about 10; C_1 to C_{10} lower alkyl, alkoxyalkoxy, substituted lower alkyl, alkaryl or aralkyl; Cl; Br; CN; CF_3 ; OCF_3 ; O-, S-, or N-alkyl; O-, S-, or N-alkenyl; SOCH_3 ; SO_2CH_3 ; ONO_2 ; NO_2 ; N_3 ; NH_2 ;
35 heterocycloalkyl; heterocycloalkaryl; aminoalkylamino;

polyalkylamino; substituted silyl; an RNA cleaving group; a reporter group; an intercalator; a group for improving the pharmacokinetic properties of an oligonucleotide; or a group for improving the pharmacodynamic properties of an oligonucleotide and other substituents having similar properties. A preferred modification includes 2'-methoxyethoxy [2'-O-CH₂CH₂OCH₃, also known as 2'-O-(2-methoxyethyl)] (Martin et al., *Helv. Chim. Acta*, 1995, 78, 486). Other preferred modifications include 2'-methoxy (2'-O-CH₃), 2'-propoxy (2'-OCH₂CH₂CH₃) and 2'-fluoro (2'-F). Similar modifications may also be made at other positions on the oligonucleotide, particularly the 3' position of the sugar on the 3' terminal nucleotide and the 5' position of 5' terminal nucleotide. Oligonucleotides may also have sugar mimetics such as cyclobutyls in place of the pentofuranosyl group.

Oligonucleotides may also include, additionally or alternatively, nucleobase (often referred to in the art simply as "base") modifications or substitutions. As used herein, "unmodified" or "natural" nucleobases include adenine (A), guanine (G), thymine (T), cytosine (C) and uracil (U). Modified nucleobases include nucleobases found only infrequently or transiently in natural nucleic acids, e.g., hypoxanthine, 6-methyladenine, 5-me pyrimidines, particularly 5-methylcytosine (also referred to as 5-methyl-2'deoxyctosine and often referred to in the art as 5-me-C), 5-hydroxymethylcytosine (HMC), glycosyl HMC and gentobiosyl HMC, as well as synthetic nucleobases, e.g., 2-aminoadenine, 2-(methylamino)adenine, 2-(imidazolylalkyl)adenine, 2-(aminoalkylamino)adenine or other heterosubstituted alkyladenines, 2-thiouracil, 2-thiothymine, 5-bromouracil, 5-hydroxymethyluracil, 8-azaguanine, 7-deazaguanine, N⁶(6-aminohexyl)adenine and 2,6-diaminopurine. Kornberg, A., *DNA Replication*, W.H. Freeman & Co., San Francisco, 1980, pp75-77; Gebeyehu, G., et al. *Nucl. Acids Res.* 1987, 15:4513). A

"universal" base known in the art, e.g., inosine, may be included. 5-me-C substitutions have been shown to increase nucleic acid duplex stability by 0.6-1.2°C (Sanghvi, Y.S., in Crooke, S.T. and Lebleu, B., eds., *Antisense Research and Applications*, CRC Press, Boca Raton, 1993, pp. 276-278) and are presently preferred base substitutions.

Another modification of the oligonucleotides of the invention involves chemically linking to the oligonucleotide one or more moieties or conjugates which enhance the activity or cellular uptake of the oligonucleotide. Such moieties include but are not limited to lipid moieties such as a cholesterol moiety, a cholesteryl moiety (Letsinger et al., *Proc. Natl. Acad. Sci. USA* 1989, 86, 6553), cholic acid (Manoharan et al. *Bioorg. Med. Chem. Lett.* 1994, 4, 1053), a thioether, e.g., hexyl-S-tritylthiol (Manoharan et al. *Ann. N.Y. Acad. Sci.* 1992, 660, 306; Manoharan et al. *Bioorg. Med. Chem. Lett.* 1993, 3, 2765), a thiocholesterol (Oberhauser et al., *Nucl. Acids Res.* 1992, 20, 533), an aliphatic chain, e.g., dodecandiol or undecyl residues (Saison-Behmoaras et al. *EMBO J.* 1991, 10, 111; Kabanov et al. *FEBS Lett.* 1990, 259, 327; Svinarchuk et al. *Biochimie* 1993, 75, 49), a phospholipid, e.g., di-hexadecyl-rac-glycerol or triethylammonium 1,2-di-O-hexadecyl-rac-glycero-3-H-phosphonate (Manoharan et al. *Tetrahedron Lett.* 1995, 36, 3651; Shea et al. *Nucl. Acids Res.* 1990, 18, 3777), a polyamine or a polyethylene glycol chain (Manoharan et al. *Nucleosides & Nucleotides* 1995, 14, 969), or adamantane acetic acid (Manoharan et al. *Tetrahedron Lett.* 1995, 36, 3651). Oligonucleotides comprising lipophilic moieties, and methods for preparing such oligonucleotides are known in the art, for example, U.S. Patents No. 5,138,045, No. 5,218,105 and No. 5,459,255.

The oligonucleotides of the invention may be provided as prodrugs, which comprise one or more moieties which are cleaved off, generally in the body, to yield an active oligonucleotide. One example of a prodrug approach is
5 described by Imbach et al. in WO Publication 94/26764.

It is not necessary for all positions in a given oligonucleotide to be uniformly modified, and in fact more than one of the aforementioned modifications may be incorporated in a single oligonucleotide or even at within a
10 single nucleoside within an oligonucleotide. The present invention also includes oligonucleotides which are chimeric oligonucleotides as hereinbefore defined.

The oligonucleotides used in accordance with this invention may be conveniently and routinely made through the
15 well-known technique of solid phase synthesis. Equipment for such synthesis is sold by several vendors including Applied Biosystems. Any other means for such synthesis may also be employed; the actual synthesis of the oligonucleotides is well within the talents of the routineer. It is also well known
20 to use similar techniques to prepare other oligonucleotides such as the phosphorothioates and alkylated derivatives. It is also well known to use similar techniques and commercially available modified amidites and controlled-pore glass (CPG) products such as biotin, fluorescein, acridine or psoralen-
25 modified amidites and/or CPG (available from Glen Research, Sterling VA) to synthesize fluorescently labeled, biotinylated or other modified oligonucleotides such as cholesterol-modified oligonucleotides.

The oligonucleotides in accordance with this invention
30 preferably comprise from about 8 to about 50 nucleic acid base units. In the context of this invention it is understood that this encompasses non-naturally occurring oligomers as hereinbefore described, having 8 to 50 monomers.

The oligonucleotides of this invention can be used in
35 diagnostics, therapeutics and as research reagents and kits. Since the oligonucleotides of this invention hybridize to the ras gene, sandwich and other assays can easily be constructed

to exploit this fact. Furthermore, since the oligonucleotides of this invention hybridize preferentially to the mutant (activated) form of the ras oncogene, such assays can be devised for screening of cells and tissues for ras conversion from wild-type to activated form. Such assays can be utilized for differential diagnosis of morphologically similar tumors, and for detection of increased risk of cancer stemming from ras gene activation. Provision of means for detecting hybridization of oligonucleotide with the ras gene can routinely be accomplished. Such provision may include enzyme conjugation, radiolabelling or any other suitable detection systems. Kits for detecting the presence or absence of nucleic acids encoding ras or activated ras may also be prepared.

The following specific descriptions serve to illustrate the invention and are not intended to limit the scope of the invention:

Antisense Oligonucleotide Inhibition of ras-Luciferase

Gene Expression: A series of antisense phosphorothioate oligonucleotides targeted to either the H-ras translation initiation codon or the codon-12 point mutation of activated H-ras were screened using the ras-luciferase reporter gene system described in Examples 2-5. Of this initial series, six oligonucleotides were identified that gave significant and reproducible inhibition of ras-luciferase activity. The base sequences, sequence reference numbers and SEQ ID numbers of these oligonucleotides (all are phosphorothioates) are shown in Table 1.

TABLE 1

OLIGO REF NO	SEQUENCE	SEQ ID NO:
2502	CTT-ATA-TTC-CGT-CAT-CGC-TC	1
5 2503	TCC-GTC-ATC-GCT-CCT-CAG-GG	2
2570	CCA-CAC-CGA-CGG-CGC-CC	3
2571	CCC-ACA-CCG-ACG-GCG-CCC-A	4
2566	GCC-CAC-ACC-GAC-GGC-GCC-CAC	5
2560	TGC-CCA-CAC-CGA-CGG-CGC-CCA-CC	6

10 A dose-response experiment was performed in which cells
expressing either the normal ras-luciferase reporter gene or
the mutant ras-luciferase reporter gene were treated with
increasing concentrations of the phosphorothioate
oligonucleotide 2503 (SEQ ID NO: 2). This compound is
15 targeted to the translational initiation codon of H-ras RNA
transcripts. Treatment of cells with this oligonucleotide
resulted in a dose-dependent inhibition of ras-luciferase
activity, displaying IC50 values of approximately 50 nM for
both the normal and the mutant ras targets. The observation
20 that an oligonucleotide targeted to the ras translation
initiation codon is equally effective in reducing both mutant
and normal ras expression is expected since the two targets
have identical sequence compositions in the region surrounding
the AUG translation initiation site.

25 Another dose-response experiment was performed in which
cells were treated with phosphorothioate oligonucleotide 2570
(SEQ ID NO: 3), a compound that is targeted to the codon-12
point mutation of mutant (activated) H-ras RNA. Treatment of
cells with increasing concentrations of this oligonucleotide
30 resulted in a dose-dependent inhibition of ras-luciferase
activity in cells expressing either the mutant form or the
normal form of ras-luciferase. However, oligonucleotide 2570
displayed approximately threefold selectivity toward the
mutant form of ras-luciferase as compared to the normal form.
35 In fact, 2570 displayed an IC50 value for the mutant form of
ras-luciferase of approximately 100 nM whereas the same

compound displayed in IC₅₀ value of approximately 250 nM for the unmutated form.

Cells expressing either the normal form or the mutant form of ras-luciferase were treated with a single dose (0.5 μ M) of oligonucleotide targeted to either the translation initiation codon of H-ras or the codon-12 point mutation. The antisense phosphorothioate oligonucleotides tested are shown in Table 1. Compound 2503 (SEQ ID NO: 2), targeted to the ras translational initiation codon, was most effective in inhibiting ras-luciferase activity, giving approximately 80% inhibition of both normal and mutant targets. ISIS 2502 gave 30-35% inhibition of both targets. Of the three compounds targeted to the codon-12 point mutation of activated H-ras, only the 17-mer oligonucleotide 2570 (SEQ ID NO: 3) displayed selectivity toward the mutated form of ras-luciferase as compared to the normal form, giving approximately 22% inhibition of the normal target and 68% inhibition of the mutant target. ISIS 2571 gave approximately 60% inhibition of both targets and ISIS 2566 gave 65-70% inhibition of both targets. Table 2 summarizes data obtained with all 13 antisense oligonucleotides targeted to H-ras. A scrambled control oligonucleotide gave no inhibition of either mutant or normal ras and a control oligonucleotide (ISIS 2907; SEQ ID NO: 19) complementary to the codon-12 region of normal ras gave 70% inhibition of the normal target but had no effect on the mutant ras. Shown for each oligonucleotide is its sequence, region to which it is complementary, and its activity in suppressing expression of the ras-luciferase fusion protein (given as IC₅₀, the concentration in nM necessary to give 50% inhibition of ras-luciferase expression). The longer phosphorothioates targeted to the codon-12 point mutation, while displaying substantial antisense activity toward ras-luciferase expression, did not demonstrate selective inhibition of expression of the mutant form of ras-luciferase. Phosphorothioate oligonucleotides targeted to the codon-12 point mutation that were less than 17 nucleotides in length did not show activity to either form

of ras-luciferase. These results demonstrate effective antisense activity of phosphorothioate oligonucleotides targeted to ras sequences.

Antisense oligonucleotides specifically hybridizable
5 with the H-ras AUG: Three 20-base phosphorothioate oligonucleotides, targeted to the H-ras AUG codon, were compared for their ability to inhibit ras-luciferase expression in transient transfection assays as described in Examples 2-5. These oligonucleotides, ISIS 2502 (SEQ ID NO:
10 1), 2503 (SEQ ID NO: 2) and 6186 (SEQ ID NO: 7) shown in Table 2, were tested for inhibition of ras-luciferase expression at a single dose (100 nM) in HeLa cells. All three AUG-targeted oligonucleotides were effective in inhibiting ras-luciferase expression. These three phosphorothioate oligonucleotides
15 were also prepared with a 2'-O-methyl modification on each sugar. The 2'-O-methylated version of ISIS 2503 (SEQ ID NO: 2) also inhibited ras-luciferase expression with an IC50 between 200 and 500 nM. SEQ ID NO: 7 as a 2'-O-methyl gave approximately 40% inhibition at the highest dose (500 nM).

TABLE 2

Antisense oligonucleotides targeted to mutant H-ras
(Oligonucleotide sequences shown 5' to 3')

	ISIS#	TARGET	SEQUENCE	IC50 (nM)	SEQ.ID NO.
5	2502	AUG	CTTATATTCCGTCATCGCTC	750	1
	2503	AUG	TCCGTCATCGCTCCTCAGGG	50	2
	6186	AUG	TATTCCGTCATCGCTCCTCA	--	7
	2563	CODON 12	CGACG	--	8
	2564	CODON 12	CCGACGG	--	9
10	2565	CODON 12	ACCGACGGC	--	10
	2567	CODON 12	CACCGACGGCG	--	11
	2568	CODON 12	ACACCGACGGCGC	--	12
	2569	CODON 12	CACACCGACGGCGCC	--	13
	3426	CODON 12	CCACACCGACGGCGCC	--	14
15	3427	CODON 12	CACACCGACGGCGCCC	--	15
	2570	CODON 12	CCACACCGACGGCGCCC	100	3
	3428	CODON 12	CCCACACCGACGGCGCCC	--	16
	3429	CODON 12	CCACACCGACGGCGCCCA	--	17
	2571	CODON 12	CCCACACCGACGGCGCCCA	250	4
20	2566	CODON 12	GCCACACCGACGGCGCCAC	250	5
	2560	CODON 12	TGCCACACCGACGGCGCCACC	750	6
	2561	CODON 12	TTGCCACACCGACGGCGCCACCA	1000	18
	2907	CODON 12 (normal)	CCACACCGCGGCGCCC	--	19

Oligonucleotide length affects antisense activity and
 25 specificity: Oligonucleotides targeted to the H-ras codon- 12
 point mutation also were effective in inhibiting expression
 of ras-luciferase. A series of eleven phosphorothioate
 oligonucleotides, ranging in length between 5 and 25 bases,
 were made and tested for ability to inhibit mutant and wild
 30 type ras-luciferase in transient transfection assays as

described in Examples 2-5. The oligonucleotides are shown in Table 2. At 100 nM oligonucleotide concentration, oligonucleotides 15 bases or greater in length were found to inhibit expression of the mutant H-ras target. Selective inhibition of mutant over wild type ras-luciferase expression was observed for oligonucleotides between 15 and 19 bases in length. The maximum selectivity observed for inhibition of mutant ras-luciferase relative to wild type was for the 17-mer 2570 (SEQ ID NO: 3) and was approximately 4-fold. In order to demonstrate that 2570 was acting in a sequence-specific manner, a variant of this compound was tested (2907; SEQ ID NO: 19) in which the central adenosine residue was replaced with cytosine, making this oligonucleotide perfectly complementary to the normal H-ras target. Hence, this oligonucleotide will contain a single mismatch at the center of the oligonucleotide/RNA duplex when fully hybridized to the mutant H-ras sequence. Oligonucleotide 2907 selectively inhibited expression of normal ras-luciferase (88% inhibition) relative to mutant ras-luciferase (5% inhibition).

Two 16-mers and two 18-mers complementary to the mutant codon-12 region (Table 2) were tested as described in Examples 2-5. Figure 1 shows the results of an experiment in which antisense activity and mutant selectivity was determined for oligonucleotides of length 13, 15, 16, 17, 18 and 19 bases in a dose-dependent manner. The results obtained with these oligonucleotides demonstrated that the compounds that were active against mutant H-ras sequences also showed selectivity; oligonucleotides of length 16 (SEQ ID NO: 14 and SEQ ID NO: 15) and 17 bases (SEQ ID NO: 3) displayed the greatest selectivity (4- and 5-fold, respectively). The 13 base compound, 2568 (SEQ ID NO: 12), did not display antisense activity at any of the tested concentrations.

Chimeric 2'-O-methyl oligonucleotides with deoxy gaps:
Based on the sequence of the mutant-selective 17-mer (2570), a series of chimeric phosphorothioate 2'-O-methyl oligonucleotides were synthesized in which the end regions consisted of 2'-O-methyl nucleosides and the central residues

formed a "deoxy gap". The number of deoxy residues ranged from zero (full 2'-O-methyl) to 17 (full deoxy). These oligonucleotides are shown in Table 3.

TABLE 3

5 Chimeric phosphorothioate oligonucleotides
 having 2'-O-methyl ends (bold) and central deoxy gap
 (Mutant codon-12 target)

	OLIGO #	DEOXY	SEQUENCE	SEQ ID NO
	4122	0	CCACACCGACGGCGCCC	3
10	3975	1	CCACACCGACGGCGCCC	3
	3979	3	CCACACCGACGGCGCCC	3
	4236	4	CCACACCGACGGCGCCC	3
	4242	4	CCACACCGACGGCGCCC	3
	3980	5	CCACACCGACGGCGCCC	3
15	3985	7	CCACACCGACGGCGCCC	3
	3984	9	CCACACCGACGGCGCCC	3
	2570	17	CCACACCGACGGCGCCC	3

These oligonucleotides were characterized for hybridization efficiency as described in Example 6, ability
20 to direct RNase H cleavage in vitro using mammalian RNase H as described in Example 8, and for antisense activity. Antisense activity against full length H-ras mRNA was determined using a transient co-transfection reporter gene system in which H-ras gene expression was monitored using a
25 ras-responsive enhancer element linked to the reporter gene luciferase, as described in Example 9.

Antisense activity of deoxy-gapped oligonucleotides against full length ras mRNA: The beneficial properties of enhanced target affinity conferred by 2'-O-methyl
30 modifications can be exploited for antisense inhibition provided these compounds are equipped with RNase H-sensitive

deoxy gaps of the appropriate length. 2'-O-methyl deoxy gap oligonucleotides were tested for antisense activity against the full length H-ras mRNA using the H-ras transactivation reporter gene system described in Example 9. Antisense experiments were performed initially at a single oligonucleotide concentration (100 nM). Chimeric 2'-O-methyl oligonucleotides containing deoxy gaps of five or more residues inhibited H-ras gene expression. The full deoxy compound gave approximately 50% inhibition. The fully 2'-O-methyl, 1-deoxy and 3-deoxy gave no inhibition. The 5-deoxy, 7-deoxy and 9-deoxy compounds gave approximately 85%, 95% and 90% inhibition, respectively. These compounds displayed activities greater than that of the full deoxy parent compound.

Dose response experiments were performed using these active compounds, along with the 2'-O-methyl chimeras containing four deoxy residues. Oligonucleotide-mediated inhibition of full-length H-ras by these oligonucleotides was dose-dependent. The most active compound was the seven-residue deoxy chimera, which displayed an activity approximately five times greater than that of the full deoxy oligonucleotide.

Shortened chimeric oligonucleotides: Enhanced target affinity conferred by the 2'-O-methyl modifications was found to confer activity on short chimeric oligonucleotides. A series of short 2'-O-methyl chimeric oligonucleotides were tested for T_m and antisense activity vs. full length ras as described in Example 9. Table 4 shows T_m s for oligonucleotides 11, 13, 15 and 17 nucleotides in length, having deoxy gaps either 5 bases long or 7 bases long. In sharp contrast to the full deoxy 13-mer, both 2'-O-methyl chimeric 13-mers inhibited ras expression, and one of the 11-mers was also active. This is shown in Figure 2.

26

TABLE 4

	LENGTH	T _m (°C)	SEQUENCE	SEQ ID NO:
	17	77.2	CCACACCGACGGCGCCC	3
	15	69.8	CACACCGACGGCGCC	13
5	13	62.1	ACACCGACGGCGC	12
	11	47.3	CACCGACGGCG	11
	17	74.6	CCACACCGACGGCGCCC	3
	15	66.2	CACACCGACGGCGCC	13
	13	58.0	ACACCGACGGCGC	12
10	11	27.7	CACCGACGGCG	11

Relative antisense activity and ability to activate RNase H cleavage in vitro by chimeric 2'-O-methyl oligonucleotides is well correlated with deoxy length (Figure 3).

15 **Asymmetrical deoxy gaps:** It is not necessary that the deoxy gap be in the center of the chimeric molecule. It was found that chimeric molecules having the nucleotides of the region at one end modified at the 2' position to enhance binding and the remainder of the molecule unmodified (2'-deoxy)
 20 can still inhibit ras expression. Oligonucleotides of SEQ ID NO: 3 (17-mer complementary to mutant codon 12) in which a 7-deoxy gap was located at either the 5' or 3' side of the 17-mer, or at different sites within the middle of the molecule, all demonstrated RNase H activation and antisense activity.
 25 However, a 5-base gap was found to be more sensitive to placement, as some gap positions rendered the duplex a poor activator of RNase H and a poor antisense inhibitor. Therefore, a 7-base deoxy gap is preferred.

Other sugar modifications: The effects of other 2' sugar
 30 modifications besides 2'-O-methyl on antisense activity in chimeric oligonucleotides have been examined. These modifications are listed in Table 5, along with the T_m values obtained when 17-mer oligonucleotides having 2'-modified nucleotides flanking a 7-base deoxy gap were hybridized with

a 25-mer oligoribonucleotide complement as described in Example 6. A relationship was observed for these oligonucleotides between alkyl length at the 2' position and T_m . As alkyl length increased, T_m decreased. The 2'-fluoro 5 chimeric oligonucleotide displayed the highest T_m of the series.

TABLE 5

Correlation of T_m with Antisense Activity
2'-modified 17-mer with 7-deoxy gap
CCACACCGACGGCGCCC (SEQ ID NO: 3)

10

2' MODIFICATION	T_m ($^{\circ}$ C)	IC50 (nM)
Deoxy	64.2	150
O-Pentyl	68.5	150
O-Propyl	70.4	70
15 O-Methyl	74.7	20
Fluoro	76.9	10

These 2' modified oligonucleotides were tested for antisense activity against H-ras using the transactivation reporter gene assay described in Example 9. As shown in Table 20 5, all of these 2' modified chimeric compounds inhibited ras expression, with the 2'-fluoro 7-deoxy-gap compound the most active. A 2'-fluoro chimeric oligonucleotide with a centered 5-deoxy gap was also active.

Chimeric phosphorothioate oligonucleotides having SEQ 25 ID NO: 3 having 2'-O-propyl regions surrounding a 5-base or 7-base deoxy gap were compared to 2'-O-methyl chimeric oligonucleotides. ras expression in T24 cells was inhibited by both 2'-O-methyl and 2'-O-propyl chimeric oligonucleotides with a 7-deoxy gap and a uniform phosphorothioate backbone. 30 When the deoxy gap was decreased to five nucleotides, only the 2'-O-methyl oligonucleotide inhibited ras expression.

Antisense oligonucleotide inhibition of H-ras gene expression in cancer cells: Two phosphorothioate oligonucleotides (2502, 2503) complementary to the ras AUG

region were tested as described in Example 10, along with chimeric oligonucleotides (4998, 5122) having the same sequence and 7-base deoxy gaps flanked by 2'-O-methyl regions. These chimeric oligonucleotides are shown in Table 6.

5

TABLE 6

Chimeric phosphorothioate oligonucleotides
having 2'-O-methyl ends (bold) and central deoxy gap
(AUG target)

	OLIGO #	DEOXY	SEQUENCE	SEQ ID NO:
10	2502	20	CTTATATTCCGTCATCGCTC	1
	4998	7	CTTATATTCCGTCATCGCTC	1
	2503	20	TCCGTCATCGCTCCTCAGGG	2
	5122	7	TCCGTCATCGCTCCTCAGGG	2

Compound 2503 inhibited ras expression in T24 cells by 71%, and the chimeric compound (4998) inhibited ras mRNA even further (84% inhibition). Compound 2502, also complementary to the AUG region, decreased ras RNA levels by 26% and the chimeric version of this oligonucleotide (5122) demonstrated 15% inhibition. Also included in this assay were two oligonucleotides targeted to the mutant codon 12. Compound 2570 (SEQ ID NO: 3) decreased ras RNA by 82% and the 2'-O-methyl chimeric version of this oligonucleotide with a seven-deoxy gap (3985) decreased ras RNA by 95%.

Oligonucleotides 2570 and 2503 were also tested to determine their effects on ras expression in HeLa cells, which have a wild-type (i.e., not activated) H-ras codon 12. While both of these oligonucleotides inhibited ras expression in T24 cells (having activated codon 12), only the oligonucleotide (2503) specifically hybridizable with the ras AUG inhibited ras expression in HeLa cells. Oligonucleotide 2570 (SEQ ID NO: 3), specifically hybridizable with the activated codon 12, did not inhibit ras expression in HeLa cells, because these cells lack the activated codon-12 target.

Oligonucleotide 2570, a 17-mer phosphorothioate oligonucleotide complementary to the codon 12 region of

activated H-ras, was tested for inhibition of ras expression (as described in Example 10) in T24 cells along with chimeric phosphorothioate 2'-O-methyl oligonucleotides 3980, 3985 and 3984, which have the same sequence as 2570 and have deoxy gaps of 5, 7 and 9 bases, respectively (shown in Table 3). The fully 2'-deoxy oligonucleotide 2570 and the three chimeric oligonucleotides decreased ras mRNA levels in T24 cells. Compounds 3985 (7-deoxy gap) and 3984 (9-deoxy gap) decreased ras mRNA by 81%; compound 3980 (5-deoxy gap) decreased ras mRNA by 61%. Chimeric oligonucleotides having this sequence, but having 2'-fluoro-modified nucleotides flanking a 5-deoxy (4689) or 7-deoxy (4690) gap, inhibited ras mRNA expression in T24 cells, with the 7-deoxy gap being preferred (82% inhibition, vs 63% inhibition for the 2'-fluoro chimera with a 5-deoxy gap).

Antisense oligonucleotide inhibition of proliferation of cancer cells: Three 17-mer oligonucleotides having the same sequence (SEQ ID NO: 3), complementary to the codon 12 region of activated ras, were tested for effects on T24 cancer cell proliferation as described in Example 11. 3985 has a 7-deoxy gap flanked by 2'-O-methyl nucleotides, and 4690 has a 7-deoxy gap flanked by 2'-F nucleotides (all are phosphorothioates). Effects of these oligonucleotides on cancer cell proliferation correlated well with their effects on ras mRNA expression shown by Northern blot analysis: oligonucleotide 2570 inhibited cell proliferation by 61%, the 2'-O-methyl chimeric oligonucleotide 3985 inhibited cell proliferation by 82%, and the 2'-fluoro chimeric analog inhibited cell proliferation by 93%.

In dose-response studies of these oligonucleotides on cell proliferation, the inhibition was shown to be dose-dependent in the 25 nM-100 nM range. IC₅₀ values of 44 nM, 61 nM and 98 nM could be assigned to oligonucleotides 4690, 3985 and 2570, respectively. The random oligonucleotide control had no effect at the doses tested.

The effect of ISIS 2570 on cell proliferation was cell type-specific. The inhibition of T24 cell proliferation by

this oligonucleotide was four times as severe as the inhibition of HeLa cells by the same oligonucleotide (100 nM oligonucleotide concentration). ISIS 2570 is targeted to the activated (mutant) ras codon 12, which is present in T24 but 5 lacking in HeLa cells, which have the wild-type codon 12.

Chimeric backbone-modified oligonucleotides:

Oligonucleotides discussed in previous examples have had uniform phosphorothioate backbones. The 2'-modified chimeric oligonucleotides discussed above are not active in uniform 10 phosphodiester backbones. A chimeric oligonucleotide was synthesized (ISIS 4226) having 2'-O-methyl regions flanking a 5-nucleotide deoxy gap, with the gap region having a P=S backbone and the flanking regions having a P=O backbone. Another chimeric oligonucleotide (ISIS 4223) having a P=O 15 backbone in the gap and P=S in flanking regions was also made. These oligonucleotides are shown in Table 7.

Additional oligonucleotides were synthesized, completely 2'-deoxy and having phosphorothioate backbones containing either a single phosphodiester (ISIS 4248), two 20 phosphodiesters (ISIS 4546), three phosphodiesters (ISIS 4551), four phosphodiesters (ISIS 4593), five phosphodiesters (ISIS 4606) or ten phosphodiester linkages (ISIS-4241) in the center of the molecule. These oligonucleotides are also shown in Table 7.

TABLE 7

Chimeric backbone (P=S/P=O) oligonucleotides
having 2'-O-methyl ends (bold) and central deoxy gap
(backbone linkages indicated by s (P=S) or o (P=O))
Mutant codon-12 target

5	OLIGO #	P=S	SEQUENCE	SEQ ID NO:
	2570	16	CsCsAsCsAsCsCsGsAsCsGsGsCsGsCsCsC	3
	4226	5	CoCoAoCoAoCsCsGsAsCsGoGoCoGoCoCoC	3
	4233	11	CsCsAsCsAsCoCoGoAoCoGsGsCsGsCsCsC	3
10	4248	15	CsCsAsCsAsCsCsGsAoCsGsGsCsGsCsCsC	3
	4546	14	CsCsAsCsAsCsCsGoAoCsGsGsCsGsCsCsC	3
	4551	13	CsCsAsCsAsCsCsGoAoCoGsGsCsGsCsCsC	3
	4593	12	CsCsAsCsAsCsCoGoAoCoGsGsCsGsCsCsC	3
	4606	11	CsCsAsCsAsCsCoGoAoCoGoGsCsGsCsCsC	3
15	4241	6	CsCsAsCoAoCoCoGoAoCoGoGoCoGsCsCsC	3

Oligonucleotides were incubated in crude HeLa cellular extracts at 37°C to determine their sensitivity to nuclease degradation as described in Dignam et al., *Nucleic Acids Res.* 1983, 11, 1475-1489. The oligonucleotide (4233) with a five-
20 diester gap between phosphorothioate/2'-O-methyl wings had a $T_{1/2}$ of 7 hr. The oligonucleotide with a five-phosphorothioate gap in a phosphorothioate/2'-O-methyl molecule had a $T_{1/2}$ of 30 hours. In the set of oligonucleotides having one to ten diester linkages, the oligonucleotide (4248) with a single
25 phosphodiester linkage was as stable to nucleases as was the full-phosphorothioate molecule, ISIS 2570, showing no degradation after 5 hours in HeLa cell extract. Oligonucleotides with two-, three- and four-diester gaps had $T_{1/2}$ of approximately 5.5 hours, 3.75 hours, and 3.2 hours, and
30 oligonucleotides with five or ten deoxy linkages had $T_{1/2}$ of 1.75 hours and 0.9 hours, respectively.

Antisense activity of chimeric backbone-modified oligonucleotides: A uniform phosphorothioate backbone is not required for antisense activity. ISIS 4226 and ISIS 4233 were tested in the ras-luciferase reporter system for effect on ras expression as described in Examples 2-5, along with ISIS 2570 (fully phosphorothioate/all deoxy), ISIS 3980 (fully phosphorothioate, 2'-O-methyl wings with deoxy gap) and ISIS 3961 (fully phosphodiester, 2'-O-methyl wings with deoxy gap). All of the oligonucleotides having a P=S (i.e., nuclease-resistant) gap region inhibited ras expression. The two completely 2'deoxy oligonucleotides having phosphorothioate backbones containing either a single phosphodiester (ISIS 4248) or ten phosphodiester linkages (ISIS 4241) in the center of the molecule were also assayed for activity. The compound containing a single P=O was just as active as a full P=S molecule, while the same compound containing ten P=O was completely inactive.

Chimeric phosphorothioate oligonucleotides of SEQ ID NO: 3 were made, having a phosphorothioate backbone in the 7-base deoxy gap region only, and phosphodiester in the flanking regions, which were either 2'-O-methyl or 2'-O-propyl. The oligonucleotide with the 2'-O-propyl diester flanking regions was able to inhibit ras expression.

Inhibition of ras-luciferase gene expression by antisense oligonucleotides containing modified bases: A series of antisense phosphorothioate oligonucleotides complementary to the codon-12 point mutation of activated ras were synthesized as described, having a 2-(amino)adenine at the position complementary to the uracil of the mutated codon 12. Because the amino group at the 2-position of the adenine is able to hydrogen bond with the oxygen in the 2-position on the uracil, three hydrogen bonds instead of the usual two are formed. This serves to greatly stabilize the hybridization of the 2-(amino)adenine-modified antisense oligonucleotide to the activated ras gene, while destabilizing or having no net effect on the stability of this oligonucleotide to the wild-

type codon 12, because of the modified A-G mismatch at this position. This increases the specificity of the modified oligonucleotide for the desired target.

An oligonucleotide having a single 2,6-
5 (diamino)adenosine at this position in an otherwise unmodified uniform phosphorothioate 17-mer (sequence identical to 2570, SEQ ID NO: 3) was found to be at least as effective an RNase H substrate as the 2570 sequence. It is therefore expected to be an effective antisense molecule. An oligonucleotide
10 having a single 2, -(diamino)adenosine at this position in a deoxy gapped phosphorothioate oligonucleotide of the same sequence also demonstrates RNase H activation.

In vivo anti-tumor data: ISIS 2503 (SEQ ID NO: 2) has been evaluated for activity against human tumors *in vivo* as
15 described in Examples 13 and 14. These studies employed a human lung adenocarcinoma cell line (A549) which was subcutaneously implanted into nude mice, resulting in tumor growth at site of implantation. Since these cells do not contain a mutation in the H-ras gene, but do express normal
20 H-ras, only the AUG-directed oligonucleotide ISIS 2503 was evaluated for anti-tumor activity.

In the first study, phosphorothioate oligonucleotides in saline were administered by intraperitoneal injection at a dosage of 20 mg/kg. Drug treatment was initiated at the
25 time tumors first became visible (28 days following tumor cell inoculation) and treatments were performed every other day. As shown in Figure 4, no effect on tumor growth was observed after treatment with the unrelated control phosphorothioate oligonucleotide ISIS 1082 (SEQ ID NO: 55). However,
30 significant inhibition of tumor growth was observed for the H-ras-specific oligonucleotide ISIS 2503 (SEQ ID NO: 2). The anti-tumor effects of the H-ras compound were first observed 20 days following initiation of drug treatment and continued throughout the duration of the study.

35 In a second study, phosphorothioate oligonucleotides were prepared in a cationic lipid formulation (DMRIE:DOPE) and administered by subcutaneous injection as described in Example

15. Drug treatment was initiated one week following tumor cell inoculation and was performed three times a week for only four weeks. As was observed in the first study, administration of the H-ras-specific compound ISIS 2503 (SEQ ID NO: 2) caused
5 a marked reduction in tumor growth whereas the unrelated control oligonucleotide (ISIS 1082) had no significant effect (Figure 5). Reduction in tumor volume was first observed 20 days following appearance of visible tumors and continued over time throughout the remainder of the study.

10 Stability of 2'-modified phosphodiester oligonucleotides in cells: Modification of oligonucleotides to confer nuclease stability is required for antisense activity in cells. Certain modifications at the 2' position of the sugar have been found to confer nuclease resistance sufficient to elicit
15 antisense effects in cells without any backbone modification. A uniformly 2'-propoxy modified phosphodiester oligonucleotide (SEQ ID NO: 3) was found to inhibit H-ras expression in T24 cells, 24 hours after administration, at a level equivalent to a phosphorothioate 2'-deoxyoligonucleotide having the same
20 sequence. Uniform 2'-methoxy phosphodiester oligonucleotide also showed some activity. 2'-pentoxy modifications were found to be at least as active as the 2'-propoxy.

Antisense oligonucleotides active against Ki-ras:

Oligonucleotides were designed to be complementary
25 to the 5'-untranslated region, 3'-untranslated region and coding region of the human Ki-ras oncogene. McGrath, J.P. et al. Nature 1983, 304, 501-506. Of the latter, oligonucleotides were targeted to codons 12 and 61 which are known sites of mutation that lead to Ki-ras-mediated
30 transformation, and also to codon 38, which is not known to be involved in transformation. The oligonucleotides are shown in Table 8.

Table 8

Antisense Oligonucleotides Complementary to Human Ki-ras

	<u>ISIS</u> <u>#</u>	<u>SEQUENCE</u>	<u>TARGET</u>	<u>SEO ID</u> <u>NO.</u>
5	6958	CTG CCT CCG CCG CCG CGG CC	5' UTR/5' cap	20
	6957	CAG TGC CTG CGC CGC GCT CG	5' -UTR	21
	6956	AGG CCT CTC TCC CGC ACC TG	5' -UTR	22
	6953	TTC AGT CAT TTT CAG CAG GC	AUG	23
	6952	TTA TAT TCA GTC ATT TTC AG	AUG	24
10	6951	CAA GTT TAT ATT CAG TCA TT	AUG	25
	6950	GCC TAC GCC ACC AGC TCC AAC	Codon 12 (WT)	26
	6949	CTA CGC CAC CAG CTC CA	Codon 12 (WT)	27
	6948	G TAC TCC TCT TGA CCT GCT GT	Codon 61 (WT)	28
	6947	CCT GTA GGA ATC CTC TAT TGT	Codon 38	29
15	6946	GGT AAT GCT AAA ACA AAT GC	3' -UTR	30
	6945	GGA ATA CTG GCA CTT CGA GG	3' -UTR	31
	7453	TAC GCC AAC AGC TCC	Codon 12 (G-T mut.)	32
	7679	TTT TCA GCA GGC CTC TCT CC	5' -UTR/AUG	33

Twelve Ki-ras-specific oligonucleotides were screened for antisense activity against three colon carcinoma cell lines that contain a mutation at codon 12 in the Ki-ras oncogene and evaluated by measurement of Ki-ras mRNA levels. As shown in Figure 6, half of the tested compounds displayed significant activity (at least 40% inhibition) against the Ki-ras transcript, with the most active compounds being targeted to the 5' - and 3' -untranslated regions. However, significant inhibition of Ki-ras expression was also observed for compounds directed against wild type codons 12 and 61. Compounds that displayed significant activity were effective against all three carcinoma cell lines tested.

Dose response analysis of these compounds demonstrated that ISIS 6958 and ISIS 6957, both of which target the 5'-UTR, are the most potent inhibitors of Ki-ras in this series of oligonucleotides. These oligonucleotides were examined for their ability to inhibit proliferation of Ki-ras transformed cell lines. The colon carcinoma cell line SW480 was treated with a single dose of oligonucleotide (200 nM) and cell number was determined over a five-day period. As shown in Figure 7 both Ki-ras specific oligonucleotides were effective inhibitors of proliferation of SW480 cells, with ISIS 6957 (SEQ ID NO: 21) showing greater activity than ISIS 6958 (SEQ ID NO: 20). This difference in activity correlates well with the inhibition of Ki-ras mRNA expression (Figure 6).

Selectivity of inhibition of mutant Ki-ras relative to normal Ki-ras: Oligonucleotides targeted to Ki-ras have been examined for their ability to selectively inhibit mutant Ki-ras relative to normal Ki-ras. Two cell lines were employed: the SW480 cell line that expresses mutant Ki-ras (codon 12, G to T transversion) and a cell line (HeLa) that expresses normal Ki-ras. Two oligonucleotides were tested: ISIS 6957, SEQ ID NO: 21, a 20mer phosphorothioate targeted to the 5'-untranslated region of Ki-ras, and ISIS 7453, SEQ ID NO: 32, a 15mer phosphorothioate targeted to the Ki-ras codon 12 region. Ki-ras mRNA levels were measured 24 hours after treatment. The codon 12-directed compound was effective in the cell line expressing mutant Ki-ras (87% inhibition vs. 18% inhibition in HeLa cells). However, the Ki-ras oligonucleotide targeted to the 5'-untranslated region was a potent inhibitor (95% inhibition) of Ki-ras expression in both cell lines. Selectivity for mutant Ki-ras was found to be dependent on oligonucleotide concentration and affinity for the RNA target.

Ki-ras oligonucleotides with deoxy gaps: Phosphorothioate oligonucleotides (SEQ ID NO: 21, targeted to the 5'-untranslated region of Ki-ras) were synthesized with 2'-O-methyl modifications flanking central 2'-deoxy gap regions of 6 or 8 nucleotides in length. Both gapped

oligonucleotides were active against Ki-ras expression as determined by Northern blot analysis. A uniformly 2'-O-methylated compound (no deoxy gap) was inactive. An additional oligonucleotide, ISIS 7679 (SEQ ID NO: 33, 5 complementary to the 5' untranslated/AUG region of Ki-ras), was also found to be active when synthesized with a 6- or 8-nucleotide deoxy gap.

2'-Methoxyethoxy analogs of ISIS 2503 (H-ras): A series of chimeric oligonucleotides were synthesized with the ISIS 10 2503 sequence (SEQ ID NO: 2) and various arrangements of 2'-methoxyethoxy (2'-MOE) modifications. These are shown in Table 9. All backbone linkages are phosphorothioates.

Table 9

2'-MOE analogs of ISIS 2503
15 Positions with 2'-MOE are shown in bold

ISIS #	Sequence (5'--3')	SEQ ID NO:
13905	TCCGTCATCGCTCCTCAGGG	2
13907	TCCGTCATCGCTCCTCAGGG	2
13909	TCCGTCATCGCTCCTCAGGG	2
20 13911	TCCGTCATCGCTCCTCAGGG	2
13917	TCCGTCATCGCTCCTCAGGG	2
13919	TCCGTCATCGCTCCTCAGGG	2
13920	TCCGTCATCGCTCCTCAGGG	2
13923	TCCGTCATCGCTCCTCAGGG	2
25 13926	TCCGTCATCGCTCCTCAGGG	2
13927	TCCGTCATCGCTCCTCAGGG	2

These oligonucleotides (except for 13919 and 13927 which have not yet been tested) were tested for the ability to reduce H-ras mRNA levels in T24 cells as described in Example 10 except 30 that oligonucleotide and lipofectin were mixed in OptiMEM and kept at a constant ratio of 2.5ug/ml lipofectin per 100 nM

oligonucleotide. All of the tested compounds had activity comparable to ISIS 2503, the parent compound, with IC50's of 50 nM or below. For this reason oligonucleotides containing one or more 2'-MOE modifications are preferred for reducing ras expression. Dose responses for these compounds are shown in Figure 8. ISIS 13177 (TCAGTAATAGCCCCACATGG; SEQ ID NO: 34) is a phosphorothioate oligodeoxynucleotide scrambled control for SEQ ID NO: 2.

MMI analogs of ISIS 2503 (H-ras): A series of chimeric oligonucleotides were synthesized with the ISIS 2503 sequence (SEQ ID NO: 2) and various placements of methylene(methylimino)backbone linkages. These are shown in Table 10. For ease of synthesis, dimers incorporating an MMI linkage were used in making these oligonucleotides. Dimers containing MMI backbone linkages are indicated by **bold** lettering. "o" indicates a phosphodiester linkage between MMI dimers. "s" indicates a phosphorothioate linkage between MMI dimers. All unmarked linkages are phosphorothioates.

Table 10

MMI analogs of ISIS 2503

ISIS #	Sequence (5'--3')	SEQ ID NO:
14896	TCCGTCATCGCTCCTCAGGG	2
14897	TC _o CGTCATCGCTCCTCAG _s GG	2
14898	TC _s CGTCATCGCTCCTCAG _s GG	2
14899	TC _o CG _s TCATCGCTCCTC _o A _s GGG	2
14900	TC _s CG _s TCATCGCTCCTC _s AG _s AG	2

These compounds were tested for their ability to reduce H-ras mRNA levels in T24 cells as described in Example 10 except that oligonucleotide and lipofectin were mixed in OptiMEM and kept at a constant ratio of 2.5 µg/ml lipofectin per 100 nM oligonucleotide. As shown in Figure 9, all of these compounds

were able to reduce mRNA levels by 80% or more at doses of 500 nM and below. ISIS 13177 (SEQ ID NO: 34) is a phosphorothioate oligodeoxynucleotide scrambled control for SEQ ID NO: 2. With the exception of ISIS 14899, all the MMI compounds were more active than the parent deoxyphosphorothioate compound, ISIS 2503. Several compounds (ISIS 14896, 14897, 14898) achieved nearly complete ablation of ras mRNA. Oligonucleotides containing one or more MMI modifications are therefore highly preferred for reducing ras expression.

Antisense oligonucleotides active against N-ras:

A series of phosphorothioate oligodeoxynucleotides were designed to target human N-ras using the published sequence (Genbank accession number HSNRASR, x02751). These compounds were tested for their ability to reduce N-ras levels in T24 cells as described in Example 10 except that the probe was an N-ras cDNA probe (purchased from Oncogene Science, Cambridge MA; catalog no. HP129) and oligonucleotide and lipofectin were mixed in OptiMEM and kept at a constant ratio of 2.5ug/ml lipofectin per 100 nM oligonucleotide.

These oligonucleotides, and the percent reduction in N-ras mRNA demonstrated for each, are shown in Table 11. Oligonucleotides shown in bold (SEQ ID NO: 44, 45, 46, 47, 49 and 52) demonstrated greater than 30% reduction of ras mRNA when screened at a 300 nM dose and are considered active in this assay. These sequences are therefore preferred. Of these oligonucleotides 14686, 14687, 14688, 14691 and 14694 (SEQ ID NO: 44, 45, 46, 49 and 52) showed greater than 50% inhibition. Dose response curves were obtained for oligonucleotides 14677, 14678, 14686, 14687, 14688, 14689, 14690, 14891, 14692, 14693, and 14694. These are shown in Figure 10. As can be seen from the figure, ISIS 14686 and ISIS 14691 (SEQ ID NO: 44 and 49, respectively) gave nearly complete ablation of N-ras mRNA at a 400 nM dose.

Table 11

Oligonucleotides targeted to human N-ras

	ISIS #	Sequence (5'--3')	Target Region	% Reduced	SEQ ID NO:
5	14677	CCGGGTCCTAGAAGCTGCAG	5' UTR	0.0	35
	14678	TAAATCAGTAAAAGAAACCG	5' UTR	0.0	36
	14679	GGACACAGTAACCAGGCGGC	5' UTR	0.0	37
	14680	AACAGAAGCTACACCAAGGG	5' UTR	0.0	38
	14681	CAGACCCATCCATTCCCGTG	5' UTR	0.0	39
10	14682	GCCAAGAAATCAGACCCATC	5' UTR	0.0	40
	14683	AGGGGGAAGATAAAACCGCC	5' UTR	0.0	41
	14684	CGCTTCCATTCTTTCGCCAT	5' UTR	0.0	42
	14685	CCGCACCCAGACCCGCCCT	5' UTR	0.0	43
	14686	CAGCCCCCACCAGGAGCGG	5' UTR	61.0	44
15	14687	GTCATTTACACCAGCAAGA	AUG	50.2	45
	14688	CAGTCATTTACACCAGCAA	AUG	60.5	46
	14689	CTCAGTCATTTACACCAGC	AUG	38.4	47
20	14690	CGTGGGCTTGTTTTGTATCA	Coding	0.2	48
	14691	CCATACAACCCTGAGTCCCA	3' UTR	58.3	49
	14692	CAGACAGCCAAGTGAGGAGG	3' UTR	0.0	50
	14693	CCAGGGCAGAAAAATAACAG	3' UTR	0.0	51
	14694	TTTGTGCTGTGGAAGAACCC	3' UTR	50.7	52
	14695	GCTATTAAATAACAATGCAC	3' UTR	0.0	53
	14696	ACTGATCACAGCTATTAAAT	3' UTR	0.0	54

The following examples illustrate the present invention
 25 and are not intended to limit the same.

EXAMPLES**Example 1 Synthesis and Characterization of Oligonucleotides**

Unmodified oligodeoxynucleotides are synthesized on an automated DNA synthesizer (Applied Biosystems model 380B) using standard phosphoramidite chemistry with oxidation by iodine. β -cyanoethyl-diisopropyl-phosphoramidites are purchased from Applied Biosystems (Foster City, CA). For phosphorothioate oligonucleotides, the standard oxidation bottle was replaced by a 0.2 M solution of ³H-1,2-benzodithiole-3-one 1,1-dioxide in acetonitrile for the stepwise thiation of the phosphite linkages. The thiation cycle wait step was increased to 68 seconds and was followed by the capping step.

2'-methoxy oligonucleotides were synthesized using 2'-methoxy β -cyanoethyl-diisopropyl-phosphoramidites (Chemgenes, Needham, MA) and the standard cycle for unmodified oligonucleotides, except the wait step after pulse delivery of tetrazole and base was increased to 360 seconds. Other 2'-alkoxy oligonucleotides were synthesized by a modification of this method, using appropriate 2'-modified amidites such as those available from Glen Research, Inc., Sterling, VA.

2'-fluoro oligonucleotides were synthesized as described in Kawasaki et al., *J. Med. Chem.* 1993, 36, 831-841. Briefly, the protected nucleoside N⁶-benzoyl-2'-deoxy-2'-fluoroadenosine was synthesized utilizing commercially available 9- β -D-arabinofuranosyladenine as starting material and by modifying literature procedures whereby the 2'- α -fluoro atom is introduced by a S_N2-displacement of a 2'- β -O-triflyl group. Thus N⁶-benzoyl-9- β -D-arabinofuranosyladenine was selectively protected in moderate yield as the 3',5'-ditetrahydropyranyl (THP) intermediate. Deprotection of the THP and N⁶-benzoyl groups was accomplished using standard methodologies and standard methods were used to obtain the 5'-

dimethoxytrityl- (DMT) and 5'-DMT-3'-phosphoramidite intermediates.

The synthesis of 2'-deoxy-2'-fluoroguanosine was accomplished using tetraisopropylidisiloxanyl (TPDS) protected 9- β -D-arabinofuranosylguanine as starting material, and conversion to the intermediate diisobutyryl-arabinofuranosylguanosine. Deprotection of the TPDS group was followed by protection of the hydroxyl group with THP to give diisobutyryl di-THP protected arabinofuranosylguanine. Selective O-deacylation and triflation was followed by treatment of the crude product with fluoride, then deprotection of the THP groups. Standard methodologies were used to obtain the 5'-DMT- and 5'-DMT-3'-phosphoramidites.

Synthesis of 2'-deoxy-2'-fluorouridine was accomplished by the modification of a known procedure in which 2, 2'-anhydro-1- β -D-arabinofuranosyluracil was treated with 70% hydrogen fluoride-pyridine. Standard procedures were used to obtain the 5'-DMT and 5'-DMT-3'phosphoramidites.

2'-deoxy-2'-fluorocytidine was synthesized via amination of 2'-deoxy-2'-fluorouridine, followed by selective protection to give N⁴-benzoyl-2'-deoxy-2'-fluorocytidine. Standard procedures were used to obtain the 5'-DMT and 5'-DMT-3'phosphoramidites.

2'-(2-methoxyethyl)-modified amidites are synthesized according to Martin, P., *Helv. Chim. Acta* 1995, 78, 486-504. For ease of synthesis, the last nucleotide was a deoxynucleotide. 2'-O-CH₂CH₂OCH₃ cytosines may be 5-methyl cytosines.

Synthesis of 5-Methyl cytosine monomers:

2,2'-Anhydro[1-(β -D-arabinofuranosyl)-5-methyluridine]:

5-Methyluridine (ribosylthymine, commercially available through Yamasa, Choshi, Japan) (72.0 g, 0.279 M), diphenylcarbonate (90.0 g, 0.420 M) and sodium bicarbonate (2.0 g, 0.024 M) were added to DMF (300 mL). The mixture was heated to reflux, with stirring, allowing the evolved carbon dioxide

gas to be released in a controlled manner. After 1 hour, the slightly darkened solution was concentrated under reduced pressure. The resulting syrup was poured into diethylether (2.5 L), with stirring. The product formed a gum. The ether was decanted and the residue was dissolved in a minimum amount of methanol (ca. 400 mL). The solution was poured into fresh ether (2.5 L) to yield a stiff gum. The ether was decanted and the gum was dried in a vacuum oven (60°C at 1 mm Hg for 24 h) to give a solid which was crushed to a light tan powder (57 g, 85% crude yield). The material was used as is for further reactions.

2'-O-Methoxyethyl-5-methyluridine:

2,2'-Anhydro-5-methyluridine (195 g, 0.81 M), tris(2-methoxyethyl)borate (231 g, 0.98 M) and 2-methoxyethanol (1.2 L) were added to a 2 L stainless steel pressure vessel and placed in a pre-heated oil bath at 160°C. After heating for 48 hours at 155-160°C, the vessel was opened and the solution evaporated to dryness and triturated with MeOH (200 mL). The residue was suspended in hot acetone (1 L). The insoluble salts were filtered, washed with acetone (150 mL) and the filtrate evaporated. The residue (280 g) was dissolved in CH₃CN (600 mL) and evaporated. A silica gel column (3 kg) was packed in CH₂Cl₂/acetone/MeOH (20:5:3) containing 0.5% Et₃NH. The residue was dissolved in CH₂Cl₂ (250 mL) and adsorbed onto silica (150 g) prior to loading onto the column. The product was eluted with the packing solvent to give 160 g (63%) of product.

2'-O-Methoxyethyl-5'-O-dimethoxytrityl-5-methyluridine:

2'-O-Methoxyethyl-5-methyluridine (160 g, 0.506 M) was co-evaporated with pyridine (250 mL) and the dried residue dissolved in pyridine (1.3 L). A first aliquot of dimethoxytrityl chloride (94.3 g, 0.278 M) was added and the mixture stirred at room temperature for one hour. A second aliquot of dimethoxytrityl chloride (94.3 g, 0.278 M) was added and the reaction stirred for an additional one hour. Methanol

(170 mL) was then added to stop the reaction. HPLC showed the presence of approximately 70% product. The solvent was evaporated and triturated with CH_3CN (200 mL). The residue was dissolved in CHCl_3 (1.5 L) and extracted with 2x500 mL of saturated NaHCO_3 and 2x500 mL of saturated NaCl . The organic phase was dried over Na_2SO_4 , filtered and evaporated. 275 g of residue was obtained. The residue was purified on a 3.5 kg silica gel column, packed and eluted with $\text{EtOAc}/\text{Hexane}/\text{Acetone}$ (5:5:1) containing 0.5% Et_3NH . The pure fractions were evaporated to give 164 g of product. Approximately 20 g additional was obtained from the impure fractions to give a total yield of 183 g (57%).

3'-O-Acetyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methyluridine:

2'-O-Methoxyethyl-5'-O-dimethoxytrityl-5-methyluridine (106 g, 0.167 M), $\text{DMF}/\text{pyridine}$ (750 mL of a 3:1 mixture prepared from 562 mL of DMF and 188 mL of pyridine) and acetic anhydride (24.38 mL, 0.258 M) were combined and stirred at room temperature for 24 hours. The reaction was monitored by tlc by first quenching the tlc sample with the addition of MeOH . Upon completion of the reaction, as judged by tlc, MeOH (50 mL) was added and the mixture evaporated at 35°C . The residue was dissolved in CHCl_3 (800 mL) and extracted with 2x200 mL of saturated sodium bicarbonate and 2x200 mL of saturated NaCl . The water layers were back extracted with 200 mL of CHCl_3 . The combined organics were dried with sodium sulfate and evaporated to give 122 g of residue (approx. 90% product). The residue was purified on a 3.5 kg silica gel column and eluted using $\text{EtOAc}/\text{Hexane}$ (4:1). Pure product fractions were evaporated to yield 96 g (84%).

3'-O-Acetyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methyl-4-triazoleuridine:

A first solution was prepared by dissolving 3'-O-acetyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methyluridine (96 g,

0.144 M) in CH₃CN (700 mL) and set aside. Triethylamine (189 mL, 1.44 M) was added to a solution of triazole (90 g, 1.3 M) in CH₃CN (1 L), cooled to -5°C and stirred for 0.5 h using an overhead stirrer. POCl₃ was added dropwise, over a 30 minute period, to the stirred solution maintained at 0-10°C, and the resulting mixture stirred for an additional 2 hours. The first solution was added dropwise, over a 45 minute period, to the later solution. The resulting reaction mixture was stored overnight in a cold room. Salts were filtered from the reaction mixture and the solution was evaporated. The residue was dissolved in EtOAc (1 L) and the insoluble solids were removed by filtration. The filtrate was washed with 1x300 mL of NaHCO₃ and 2x300 mL of saturated NaCl, dried over sodium sulfate and evaporated. The residue was triturated with EtOAc to give the title compound.

2'-O-Methoxyethyl-5'-O-dimethoxytrityl-5-methylcytidine:

A solution of 3'-O-acetyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methyl-4-triazoleuridine (103 g, 0.141 M) in dioxane (500 mL) and NH₄OH (30 mL) was stirred at room temperature for 2 hours. The dioxane solution was evaporated and the residue azeotroped with MeOH (2x200 mL). The residue was dissolved in MeOH (300 mL) and transferred to a 2 liter stainless steel pressure vessel. MeOH (400 mL) saturated with NH₃ gas was added and the vessel heated to 100°C for 2 hours (tlc showed complete conversion). The vessel contents were evaporated to dryness and the residue was dissolved in EtOAc (500 mL) and washed once with saturated NaCl (200 mL). The organics were dried over sodium sulfate and the solvent was evaporated to give 85 g (95%) of the title compound.

N'-Benzoyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methylcytidine:

2'-O-Methoxyethyl-5'-O-dimethoxytrityl-5-methylcytidine (85 g, 0.134 M) was dissolved in DMF (800 mL) and benzoic anhydride (37.2 g, 0.165 M) was added with stirring. After

stirring for 3 hours, tlc showed the reaction to be approximately 95% complete. The solvent was evaporated and the residue azeotroped with MeOH (200 mL). The residue was dissolved in CHCl₃ (700 mL) and extracted with saturated NaHCO₃ (2x300 mL) and saturated NaCl (2x300 mL), dried over MgSO₄ and evaporated to give a residue (96 g). The residue was chromatographed on a 1.5 kg silica column using EtOAc/Hexane (1:1) containing 0.5% Et₃NH as the eluting solvent. The pure product fractions were evaporated to give 90 g (90%) of the title compound.

N'-Benzoyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methylcytidine-3'-amidite:

N'-Benzoyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methylcytidine (74 g, 0.10 M) was dissolved in CH₂Cl₂ (1 L). Tetrazole diisopropylamine (7.1 g) and 2-cyanoethoxy-tetra-(isopropyl)phosphite (40.5 mL, 0.123 M) were added with stirring, under a nitrogen atmosphere. The resulting mixture was stirred for 20 hours at room temperature (tlc showed the reaction to be 95% complete). The reaction mixture was extracted with saturated NaHCO₃ (1x300 mL) and saturated NaCl (3x300 mL). The aqueous washes were back-extracted with CH₂Cl₂ (300 mL), and the extracts were combined, dried over MgSO₄, and concentrated. The residue obtained was chromatographed on a 1.5 kg silica column using EtOAc/Hexane (3:1) as the eluting solvent. The pure fractions were combined to give 90.6 g (87%) of the title compound.

5-methyl-2'-deoxycytidine (5-me-C) containing oligonucleotides were synthesized according to published methods (Sanghvi et al. *Nucl. Acids Res.* **1993**, 21, 3197-3203) using commercially available phosphoramidites (Glen Research, Sterling VA or ChemGenes, Needham MA).

Oligonucleotides having methylene(methylimino) (MMI) backbones are synthesized according to U.S. Patent 5,378,825, which is coassigned to the assignee of the present invention and is incorporated herein in its entirety. For ease of

synthesis, various nucleoside dimers containing MMI linkages were synthesized and incorporated into oligonucleotides. Other nitrogen-containing backbones are synthesized according to WO 92/20823 which is also coassigned to the assignee of the present invention and incorporated herein in its entirety.

Oligonucleotides having amide backbones are synthesized according to De Mesmaeker et al. *Acc. Chem. Res.* **1995**, 28, 366-374. The amide moiety is readily accessible by simple and well-known synthetic methods and is compatible with the conditions required for solid phase synthesis of oligonucleotides.

Oligonucleotides with morpholino backbones are synthesized according to U.S. Patent 5,034,506 (Summerton and Weller).

Peptide-nucleic acid (PNA) oligomers are synthesized according to P.E. Nielsen et al. *Science* **1991**, 254, 1497).

After cleavage from the controlled pore glass column (Applied Biosystems) and deblocking in concentrated ammonium hydroxide at 55°C for 18 hours, the oligonucleotides are purified by precipitation twice out of 0.5 M NaCl with 2.5 volumes ethanol. Synthesized oligonucleotides were analyzed by polyacrylamide gel electrophoresis on denaturing gels and judged to be at least 85% full length material. The relative amounts of phosphorothioate and phosphodiester linkages obtained in synthesis were periodically checked by ³¹P nuclear magnetic resonance spectroscopy, and for some studies oligonucleotides were purified by HPLC, as described by Chiang et al. *J. Biol. Chem.* **1991**, 266:18162-18171. Results obtained with HPLC-purified material were similar to those obtained with non-HPLC purified material.

Example 2 ras-Luciferase Reporter Gene Assembly

The ras-luciferase reporter genes described in this study were assembled using PCR technology. Oligonucleotide primers were synthesized for use as primers for PCR cloning

of the 5'-regions of exon 1 of both the mutant (codon 12) and non-mutant (wild-type) human H-ras genes. The plasmids pT24-C3, containing the c-H-ras1 activated oncogene (codon 12, GGC-GTC), and pbc-N1, containing the c-H-ras proto-oncogene, were obtained from the American Type Culture Collection (Bethesda, MD). The plasmid pT3/T7 luc, containing the 1.9 kb firefly luciferase gene, was obtained from Clontech Laboratories (Palo Alto, CA). The oligonucleotide PCR primers were used in standard PCR reactions using mutant and non-mutant H-ras genes as templates. These primers produce a DNA product of 145 base pairs corresponding to sequences -53 to +65 (relative to the translational initiation site) of normal and mutant H-ras, flanked by NheI and HindIII restriction endonuclease sites. The PCR product was gel purified, precipitated, washed and resuspended in water using standard procedures.

PCR primers for the cloning of the *P. pyralis* (firefly) luciferase gene were designed such that the PCR product would code for the full-length luciferase protein with the exception of the amino-terminal methionine residue, which would be replaced with two amino acids, an amino-terminal lysine residue followed by a leucine residue. The oligonucleotide PCR primers used for the cloning of the luciferase gene were used in standard PCR reactions using a commercially available plasmid (pT3/T7-Luc) (Clontech), containing the luciferase reporter gene, as a template. These primers yield a product of approximately 1.9 kb corresponding to the luciferase gene, flanked by unique HindIII and BssHII restriction endonuclease sites. This fragment was gel purified, precipitated, washed and resuspended in water using standard procedures.

To complete the assembly of the ras-luciferase fusion reporter gene, the ras and luciferase PCR products were digested with the appropriate restriction endonucleases and cloned by three-part ligation into an expression vector containing the steroid-inducible mouse mammary tumor virus

promotor MMTV using the restriction endonucleases NheI, HindIII and BssHII. The resulting clone results in the insertion of H-ras 5' sequences (-53 to +65) fused in frame with the firefly luciferase gene. The resulting expression
5 vector encodes a ras-luciferase fusion product which is expressed under control of the steroid-inducible MMTV promoter. These plasmid constructions contain sequences encoding amino acids 1-22 of activated (RA2) or normal (RA4) H-ras proteins fused in frame with sequences coding for
10 firefly luciferase. Translation initiation of the ras-luciferase fusion mRNA is dependent upon the natural H-ras AUG codon. Both mutant and normal H-ras luciferase fusion constructions were confirmed by DNA sequence analysis using standard procedures.

15 Example 3 Transfection of Cells with Plasmid DNA

Transfections were performed as described by Greenberg, M.E., in *Current Protocols in Molecular Biology*, (F.M. Ausubel, R. Brent, R.E. Kingston, D.D. Moore, J.A. Smith, J.G. Seidman and K. Strahl, eds.), John Wiley and Sons, NY, with
20 the following modifications. HeLa cells were plated on 60 mm dishes at 5×10^5 cells/dish. A total of 10 μ g or 12 μ g of DNA was added to each dish, of which 1 μ g was a vector expressing the rat glucocorticoid receptor under control of the constitutive Rous sarcoma virus (RSV) promoter and the
25 remainder was ras-luciferase reporter plasmid. Calcium phosphate-DNA coprecipitates were removed after 16-20 hours by washing with Tris-buffered saline [50 mM Tris-Cl (pH 7.5), 150 mM NaCl] containing 3 mM EGTA. Fresh medium supplemented with 10% fetal bovine serum was then added to the cells. At
30 this time, cells were pre-treated with antisense oligonucleotides prior to activation of reporter gene expression by dexamethasone.

Example 4 Oligonucleotide Treatment of Cells

Following plasmid transfection, cells were washed with phosphate buffered saline prewarmed to 37°C and Opti-MEM containing 5 µg/mL N-[1-(2,3-dioleyloxy)propyl]-N,N,N,-
5 trimethylammonium chloride (DOTMA) was added to each plate (1.0 ml per well). Oligonucleotides were added from 50 µM stocks to each plate and incubated for 4 hours at 37°C. Medium was removed and replaced with DMEM containing 10% fetal bovine serum and the appropriate oligonucleotide at the
10 indicated concentrations and cells were incubated for an additional 2 hours at 37°C before reporter gene expression was activated by treatment of cells with dexamethasone to a final concentration of 0.2 µM. Cells were harvested and assayed for luciferase activity fifteen hours following dexamethasone
15 stimulation.

Example 5 Luciferase Assays

Luciferase was extracted from cells by lysis with the detergent Triton X-100 as described by Greenberg, M.E., in *Current Protocols in Molecular Biology*, (F.M. Ausubel, R.
20 Brent, R.E. Kingston, D.D. Moore, J.A. Smith, J.G. Seidman and K. Strahl, eds.), John Wiley and Sons, NY. A Dynatech ML1000 luminometer was used to measure peak luminescence upon addition of luciferin (Sigma) to 625 µM. For each extract, luciferase assays were performed multiple times, using
25 differing amounts of extract to ensure that the data were gathered in the linear range of the assay.

Example 6 Melting Curves

Absorbance vs temperature curves were measured at 260 nm using a Gilford 260 spectrophotometer interfaced to an IBM
30 PC computer and a Gilford Response II spectrophotometer. The buffer contained 100 mM Na⁺, 10 mM phosphate and 0.1 mM EDTA, pH 7. Oligonucleotide concentration was 4 µM each strand determined from the absorbance at 85°C and extinction

coefficients calculated according to Puglisi and Tinoco, *Methods in Enzymol.* **1989**, 180, 304-325. T_m values, free energies of duplex formation and association constants were obtained from fits of data to a two state model with linear
5 sloping baselines. Petersheim, M. and Turner, D.H., *Biochemistry* **1983**, 22, 256-263. Reported parameters are averages of at least three experiments. For some oligonucleotides, free energies of duplex formation were also obtained from plots of T_m^{-1} vs \log_{10} (concentration). Borer,
10 P.N., Dengler, B., Tinoco, I., Jr., and Uhlenbeck, O.C., *J. Mol. Biol.*, **1974**, 86, 843-853.

Example 7 Gel Shift Assay

The structured ras target transcript, a 47-nucleotide hairpin containing the mutated codon 12, was prepared and
15 mapped as described in Lima et al., *Biochemistry* **1992**, 31, 12055-12061. Hybridization reactions were prepared in 20 μ l containing 100 mM sodium, 10 mM phosphate, 0.1 mM EDTA, 100 CPM of T7-generated RNA (approximately 10 pM), and antisense oligonucleotide ranging in concentration from 1 pM to 10 μ M.
20 Reactions were incubated 24 hours at 37°C. Following hybridization, loading buffer was added to the reactions and reaction products were resolved on 20% native polyacrylamide gels, prepared using 45 mM tris-borate and 1 mM $MgCl_2$ (TBM). Electrophoresis was carried out at 10°C and gels were
25 quantitated using a Molecular Dynamics Phosphorimager.

Example 8 RNase H Analysis

RNase H assays were performed using a chemically synthesized 25-base oligoribonucleotide corresponding to bases +23 to +47 of activated (codon 12, G-U) H-ras mRNA. The 5'
30 end-labeled RNA was used at a concentration of 20 nM and incubated with a 10-fold molar excess of antisense oligonucleotide in a reaction containing 20 mM Tris-Cl, pH

7.5, 100 mM KCl, 10 mM MgCl₂, 1 mM dithiothreitol, 10 µg tRNA and 4 U RNasin in a final volume of 10 µl. The reaction components were preannealed at 37°C for 15 minutes then allowed to cool slowly to room temperature. HeLa cell nuclear
5 extracts were used as a source of mammalian RNase H. Reactions were initiated by addition of 2 µg of nuclear extract (5 µl) and reactions were allowed to proceed for 10 minutes at 37°C. Reactions were stopped by phenol/chloroform extraction and RNA components were precipitated with ethanol.
10 Equal CPMs were loaded on a 20% polyacrylamide gel containing 7M urea and RNA cleavage products were resolved and visualized by electrophoresis followed by autoradiography. Quantitation of cleavage products was performed using a Molecular Dynamics Densitometer.

15 **Example 9 ras Transactivation Reporter Gene System**

The expression plasmid pSV2-oli, containing an activated (codon 12, GGC-GTC) H-ras cDNA insert under control of the constitutive SV40 promoter, was a gift from Dr. Bruno Tocque (Rhone-Poulenc Sante, Vitry, France). This plasmid was used
20 as a template to construct, by PCR, a H-ras expression plasmid under regulation of the steroid-inducible mouse mammary tumor virus (MMTV) promoter. To obtain H-ras coding sequences, the 570 bp coding region of the H-ras gene was amplified by PCR. The PCR primers were designed with unique restriction
25 endonuclease sites in their 5'-regions to facilitate cloning. The PCR product containing the coding region of the H-ras codon 12 mutant oncogene was gel purified, digested, and gel purified once again prior to cloning. This construction was completed by cloning the insert into the expression plasmid
30 pMAMneo (Clontech Laboratories, CA).

The ras-responsive reporter gene pRDO53 was used to detect ras expression. Owen et al., *Proc. Natl. Acad. Sci. U.S.A.* 1990, 87, 3866-3870.

Example 10

Northern blot analysis of ras
expression in vivo

The human urinary bladder cancer cell line T24 was obtained from the American Type Culture Collection (Rockville MD). Cells were grown in McCoy's 5A medium with L-glutamine (Gibco BRL, Gaithersburg MD), supplemented with 10% heat-inactivated fetal calf serum and 50 U/ml each of penicillin and streptomycin. Cells were seeded on 100 mm plates. When they reached 70% confluency, they were treated with oligonucleotide. Plates were washed with 10 ml prewarmed PBS and 5 ml of Opti-MEM reduced-serum medium containing 2.5 μ l DOTMA was added. Oligonucleotide was then added to the desired concentration. After 4 hours of treatment, the medium was replaced with McCoy's medium. Cells were harvested 48 hours after oligonucleotide treatment and RNA was isolated using a standard CsCl purification method. Kingston, R.E., in *Current Protocols in Molecular Biology*, (F.M. Ausubel, R. Brent, R.E. Kingston, D.D. Moore, J.A. Smith, J.G. Seidman and K. Strahl, eds.), John Wiley and Sons, NY.

The human epithelioid carcinoma cell line HeLa 229 was obtained from the American Type Culture Collection (Bethesda, MD). HeLa cells were maintained as monolayers on 6-well plates in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and 100 U/ml penicillin. Treatment with oligonucleotide and isolation of RNA were essentially as described above for T24 cells.

Northern hybridization: 10 μ g of each RNA was electrophoresed on a 1.2% agarose/formaldehyde gel and transferred overnight to GeneBind 45 nylon membrane (Pharmacia LKB, Piscataway, NJ) using standard methods. Kingston, R.E., in *Current Protocols in Molecular Biology*, (F.M. Ausubel, R. Brent, R.E. Kingston, D.D. Moore, J.A. Smith, J.G. Seidman and K. Strahl, eds.), John Wiley and Sons, NY. RNA was UV-crosslinked to the membrane. Double-stranded 32 P-labeled probes were synthesized using the Prime a Gene labeling kit

(Promega, Madison WI). The ras probe was a SalI-NheI fragment of a cDNA clone of the activated (mutant) H-ras mRNA having a GGC-to-GTC mutation at codon-12. The control probe was G3PDH. Blots were prehybridized for 15 minutes at 68°C with the QuickHyb hybridization solution (Stratagene, La Jolla, CA). The heat-denatured radioactive probe (2.5×10^6 counts/2 ml hybridization solution) mixed with 100 μ l of 10 mg/ml salmon sperm DNA was added and the membrane was hybridized for 1 hour at 68°C. The blots were washed twice for 15 minutes at room temperature in 2x SSC/0.1% SDS and once for 30 minutes at 60°C with 0.1xSSC/0.1%SDS. Blots were autoradiographed and the intensity of signal was quantitated using an ImageQuant PhosphorImager (Molecular Dynamics, Sunnyvale, CA). Northern blots were first hybridized with the ras probe, then stripped by boiling for 15 minutes in 0.1x SSC/0.1%SDS and rehybridized with the control G3PDH probe to check for correct sample loading.

Example 11 Antisense oligonucleotide inhibition of proliferation of cancer cells

Cells were cultured and treated with oligonucleotide essentially as described in Example 10. Cells were seeded on 60 mm plates and were treated with oligonucleotide in the presence of DOTMA when they reached 70% confluency.

Time course experiment: On day 1, cells were treated with a single dose of oligonucleotide at a final concentration of 100 nM. The growth medium was changed once on day 3 and cells were counted every day for 5 days, using a counting chamber. Dose-response experiment: Various concentrations of oligonucleotide (10, 25, 50, 100 or 250 nM) were added to the cells and cells were harvested and counted 3 days later. Oligonucleotides 2570, 3985 and 4690 were tested for effects on T24 cancer cell proliferation.

**Example 12 Synthesis of 2-(amino)adenine-substituted
oligonucleotides**

Oligonucleotides are synthesized as in Example 1, with the following exception: at positions at which a 2-
5 (amino)adenine is desired, the standard phosphoramidite is replaced with a commercially available 2-aminodeoxyadenosine phosphoramidite (Chemgenes).

Example 13 Culture of A549 cells

A549 cells (obtained from the American Type Culture
10 Collection, Bethesda MD) were grown to confluence in 6-well plates (Falcon Labware, Lincoln Park, NJ) in Dulbecco's modified Eagle's medium (DME) containing 1 g glucose/liter and 10% fetal calf serum (FCS, Irvine Scientific, Santa Ana, CA).

**Example 14 Oligonucleotide treatment of human tumor
15 cells in nude mice - intraperitoneal
injection**

Human lung carcinoma A549 cells were harvested and 5×10^6 cells (200 μ l) were injected subcutaneously into the inner thigh of nude mice. Palpable tumors develop in approximately
20 one month. Phosphorothioate oligonucleotides ISIS 2503 and 1082 (unrelated control) were administered to mice intraperitoneally at a dosage of 20 mg/kg body weight, every other day for approximately ten weeks. Mice were monitored for tumor growth during this time.

**25 Example 15 Oligonucleotide treatment of human tumor
cells in nude mice - subcutaneous injection
with cationic lipid**

Human lung carcinoma A549 cells were harvested and 5×10^6 cells (200 μ l) were injected subcutaneously into the inner
30 thigh of nude mice. Palpable tumors develop in approximately one month. Phosphorothioate oligonucleotides ISIS 2503 and the unrelated control oligonucleotide 1082 (dosage 5 mg/kg), prepared in a cationic lipid formulation (DMRIE/DOPE, 60

mg/kg) were administered to mice subcutaneously at the tumor site. Drug treatment began one week following tumor cell inoculation and was given twice a week for only four weeks. Mice were monitored for tumor growth for a total of nine weeks.

Example 16 Stability of 2' modified oligonucleotides in T24 cells

T24 bladder cancer cells were grown as described in Example 10. Cells were treated with a single dose (1 μ M) of oligonucleotide and assayed for H-ras mRNA expression by Northern blot analysis 24 hours later. Oligonucleotides tested were analogs of ISIS 2570 (SEQ ID NO: 3), a 17mer targeted to H-ras codon 12.

Example 17 Activity of Ki-ras oligonucleotides against three colon carcinoma cell lines

Human colon carcinoma cell lines Calu 1, SW480 and SW620 were obtained from the American Type Culture Collection (ATCC) and cultured and maintained as described for HeLa cells in Example 10. Cells were treated with a single dose of oligonucleotide (200 nM) and Ki-ras mRNA expression was measured by Northern blot analysis 24 hours later. For proliferation studies, cells were treated with a single dose of oligonucleotide (200 nM) at day zero and cell number was monitored over a five-day period.

**Example 18 Oligonucleotide inhibition of mutant vs.
wild-type Ki-ras**

SW480 cells were cultured as in the previous example.
HeLa cells were cultured as in Example 10. Cells were treated
5 with a single dose (100 nM) of oligonucleotide and mRNA levels
were determined by Northern blot analysis 24 hours later.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Monia, B.P., Cowsert, L.M. and Manoharan, M.

(ii) TITLE OF INVENTION: Antisense Oligonucleotide
Inhibition of ras

(iii) NUMBER OF SEQUENCES: 55

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(F) ZIP: 08053

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: DISKETTE, 3.5 INCH, 1.44 Mb STORAGE

(B) COMPUTER: IBM PS/2

(C) OPERATING SYSTEM: Pentium

(D) SOFTWARE: WORDPERFECT 6.1

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER: not assigned

(B) FILING DATE: herewith

(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA 1:

(A) APPLICATION NUMBER: 08/411,734

(B) FILING DATE: April 3, 1995

(vii) PRIOR APPLICATION DATA 2:

59

- (A) APPLICATION NUMBER: PCT/US93/09346
- (B) FILING DATE: October 1, 1993

(vii) PRIOR APPLICATION DATA 3:

- (A) APPLICATION NUMBER: 958,134
- (B) FILING DATE: October 5, 1992

(vii) PRIOR APPLICATION DATA 4:

- (A) APPLICATION NUMBER: 08/007,996
- (B) FILING DATE: January 21, 1993

(vii) PRIOR APPLICATION DATA 5:

- (A) APPLICATION NUMBER: 08/889,296
- (B) FILING DATE: July 8, 1997

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(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20
- (B) TYPE: Nucleic Acid
- (C) STRANDEDNESS: Single
- (D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

60

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

CTTATATTCC GTCATCGCTC 20

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

TCCGTCATCG CTCCTCAGGG 20

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 17

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

CCACACCGAC GGCGCCC 17

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 19

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

61

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

CCCACACCGA CGGCGCCCA 19

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

GCCCACACCG ACGGCGCCCA C 21

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 23

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

TGCCCACACC GACGGCGCCC ACC 23

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single

62

(D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

TATTCCGTCA TCGCTCCTCA 20

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 5

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

CGACG 5

(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 7

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

CCGACGG 7

(2) INFORMATION FOR SEQ ID NO: 10:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 9

(B) TYPE: Nucleic Acid

63

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

ACCGACGGC 9

(2) INFORMATION FOR SEQ ID NO: 11:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 11

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

CACCGACGGC G 11

(2) INFORMATION FOR SEQ ID NO: 12:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 13

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

ACACCGACGG CGC 13

(2) INFORMATION FOR SEQ ID NO: 13:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 15

64

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

CACACCGACG GCGCC 15

(2) INFORMATION FOR SEQ ID NO: 14:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 16

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

CCACACCGAC GGCGCC 16

(2) INFORMATION FOR SEQ ID NO: 15:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 16

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:

CACACCGACG GCGCCC 16

(2) INFORMATION FOR SEQ ID NO: 16:

(i) SEQUENCE CHARACTERISTICS:

65

- (A) LENGTH: 18
- (B) TYPE: Nucleic Acid
- (C) STRANDEDNESS: Single
- (D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 16:

CCCACACCGA CGGCGCCC 18

(2) INFORMATION FOR SEQ ID NO: 17:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 18
- (B) TYPE: Nucleic Acid
- (C) STRANDEDNESS: Single
- (D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 17:

CCACACCGAC GCGGCCCA 18

(2) INFORMATION FOR SEQ ID NO: 18:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 25
- (B) TYPE: Nucleic Acid
- (C) STRANDEDNESS: Single
- (D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 18:

TTGCCACAC CGACGGCGCC CACCA 25

(2) INFORMATION FOR SEQ ID NO: 19:

66

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 17
- (B) TYPE: Nucleic Acid
- (C) STRANDEDNESS: Single
- (D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 19:

CCACACCGCC GCGCCC 17

(2) INFORMATION FOR SEQ ID NO: 20:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20
- (B) TYPE: Nucleic Acid
- (C) STRANDEDNESS: Single
- (D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 20

CTGCCTCCGC CGCCGCGGCC 20

(2) INFORMATION FOR SEQ ID NO: 21:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20
- (B) TYPE: Nucleic Acid
- (C) STRANDEDNESS: Single
- (D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 21:

67

CAGTGCCTGC GCCGCGCTCG 20

(2) INFORMATION FOR SEQ ID NO: 22:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20
- (B) TYPE: Nucleic Acid
- (C) STRANDEDNESS: Single
- (D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 22:

AGGCCTCTCT CCCGCACCTG 20

(2) INFORMATION FOR SEQ ID NO: 23:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20
- (B) TYPE: Nucleic Acid
- (C) STRANDEDNESS: Single
- (D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 23:

TTCAGTCATT TTCAGCAGGC 20

(2) INFORMATION FOR SEQ ID NO: 24:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20
- (B) TYPE: Nucleic Acid
- (C) STRANDEDNESS: Single
- (D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

68

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 24:

TTATATTCAG TCATTTTCAG 20

(2) INFORMATION FOR SEQ ID NO: 25:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 25:

CAAGTTTATA TTCAGTCATT 20

(2) INFORMATION FOR SEQ ID NO: 26:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 26:

GCCTACGCCA CCAGCTCCAA C 21

(2) INFORMATION FOR SEQ ID NO: 27:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 17

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

69

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 27:

CTACGCCACC AGCTCCA 17

(2) INFORMATION FOR SEQ ID NO: 28:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 28:

GTACTCCTCT TGACCTGCTG T 21

(2) INFORMATION FOR SEQ ID NO: 29:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 29:

CCTGTAGGAA TCCTCTATTG T 21

(2) INFORMATION FOR SEQ ID NO: 30:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single

70

(D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 30:

GGTAATGCTA AAACAAATGC 20

(2) INFORMATION FOR SEQ ID NO: 31:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 31:

GGAATACTGG CACTTCGAGG 20

(2) INFORMATION FOR SEQ ID NO: 32:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 15

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 32:

TACGCCAACA GCTCC 15

(2) INFORMATION FOR SEQ ID NO: 33:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20

(B) TYPE: Nucleic Acid

71

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 33:

TTTTTCAGCAG GCCTCTCTCC 20

(2) INFORMATION FOR SEQ ID NO: 34:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 34:

TCAGTAATAG CCCCACATGG 20

(2) INFORMATION FOR SEQ ID NO: 35:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 35:

CCGGGTCCTA GAAGCTGCAG 20

(2) INFORMATION FOR SEQ ID NO: 36:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20

72

- (B) TYPE: Nucleic Acid
- (C) STRANDEDNESS: Single
- (D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 36:

TAAATCAGTA AAAGAAACCG 20

(2) INFORMATION FOR SEQ ID NO: 37:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20
- (B) TYPE: Nucleic Acid
- (C) STRANDEDNESS: Single
- (D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 37:

GGACACAGTA ACCAGGCGGC 20

(2) INFORMATION FOR SEQ ID NO: 38:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20
- (B) TYPE: Nucleic Acid
- (C) STRANDEDNESS: Single
- (D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 38:

AACAGAAGCT ACACCAAGGG 20

(2) INFORMATION FOR SEQ ID NO: 39:

(i) SEQUENCE CHARACTERISTICS:

73

(A) LENGTH: 20

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 39:

CAGACCCATC CATTCCCGTG 20

(2) INFORMATION FOR SEQ ID NO: 40:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 40:

GCCAAGAAAT CAGACCCATC 20

(2) INFORMATION FOR SEQ ID NO: 41:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 41:

AGGGGGAAGA TAAAACCGCC 20

(2) INFORMATION FOR SEQ ID NO: 42:

74

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20
- (B) TYPE: Nucleic Acid
- (C) STRANDEDNESS: Single
- (D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 42:

CGCTTCCATT CTTTCGCCAT 20

(2) INFORMATION FOR SEQ ID NO: 43:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20
- (B) TYPE: Nucleic Acid
- (C) STRANDEDNESS: Single
- (D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 43:

CCGCACCCAG ACCCGCCCCT 20

(2) INFORMATION FOR SEQ ID NO: 44:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20
- (B) TYPE: Nucleic Acid
- (C) STRANDEDNESS: Single
- (D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 44:

CAGCCCCAC CAAGGAGCGG 20

75

(2) INFORMATION FOR SEQ ID NO: 45:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20
- (B) TYPE: Nucleic Acid
- (C) STRANDEDNESS: Single
- (D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 45:

GTCATTTAC ACCAGCAAGA 20

(2) INFORMATION FOR SEQ ID NO: 46:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20
- (B) TYPE: Nucleic Acid
- (C) STRANDEDNESS: Single
- (D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 46:

CAGTCATTTC ACACCAGCAA 20

(2) INFORMATION FOR SEQ ID NO: 47:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20
- (B) TYPE: Nucleic Acid
- (C) STRANDEDNESS: Single
- (D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 47:

76

CTCAGTCATT TCACACCAGC 20

(2) INFORMATION FOR SEQ ID NO: 48:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20
- (B) TYPE: Nucleic Acid
- (C) STRANDEDNESS: Single
- (D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 48:

CGTGGGCTTG TTTGTATCA 20

(2) INFORMATION FOR SEQ ID NO: 49:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20
- (B) TYPE: Nucleic Acid
- (C) STRANDEDNESS: Single
- (D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 49:

CCATACAACC CTGAGTCCCA 20

(2) INFORMATION FOR SEQ ID NO: 50:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20
- (B) TYPE: Nucleic Acid
- (C) STRANDEDNESS: Single
- (D) TOPOLOGY: Linear

77

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 50:

CAGACAGCCA AGTGAGGAGG 20

(2) INFORMATION FOR SEQ ID NO: 51:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 51:

CCAGGGCAGA AAAATAACAG 20

(2) INFORMATION FOR SEQ ID NO: 52:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 52:

TTTGTGCTGT GGAAGAACCC 20

(2) INFORMATION FOR SEQ ID NO: 53:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single

78

(D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 53:

GCTATTAAAT AACAATGCAC 20

(2) INFORMATION FOR SEQ ID NO: 54:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 54:

ACTGATCACA GCTATTAAAT 20

(2) INFORMATION FOR SEQ ID NO: 55:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 55:

GCCGAGGTCC ATGTCGTACG C 21

What is claimed is:

1. An oligonucleotide 8 to 30 nucleotides in length which is targeted to a nucleic acid encoding human N-ras and which is capable of inhibiting human N-ras expression.
- 5 2. The oligonucleotide of claim 1 which is targeted to a 5' untranslated region, translation initiation site, coding region or 3' untranslated region of an mRNA encoding human N-ras.
3. The oligonucleotide of claim 1 which has SEQ ID NO:
10 44, 45, 46, 47, 49 or 52.
4. The oligonucleotide of claim 1 which comprises at least one backbone modification.
5. The oligonucleotide of claim 1 wherein at least one of the nucleotide units of the oligonucleotide is modified at
15 the 2' position of the sugar.
6. The oligonucleotide of claim 1 in a pharmaceutically acceptable carrier.
7. A method of modulating the expression of human N-ras comprising contacting tissues or cells containing a human
20 N-ras gene with an effective amount of an oligonucleotide of claim 1, whereby expression of human N-ras is modulated.
8. A method of inhibiting the proliferation of cancer cells comprising contacting cancer cells with an effective amount of an oligonucleotide of claim 1, whereby proliferation
25 of the cancer cells is inhibited.
9. A method of preventing or treating a condition arising from the activation of a human N-ras oncogene comprising contacting an animal suspected of having a

condition arising from the activation of a human N-ras oncogene with an effective amount of an oligonucleotide of claim 1, whereby said condition is prevented or treated.

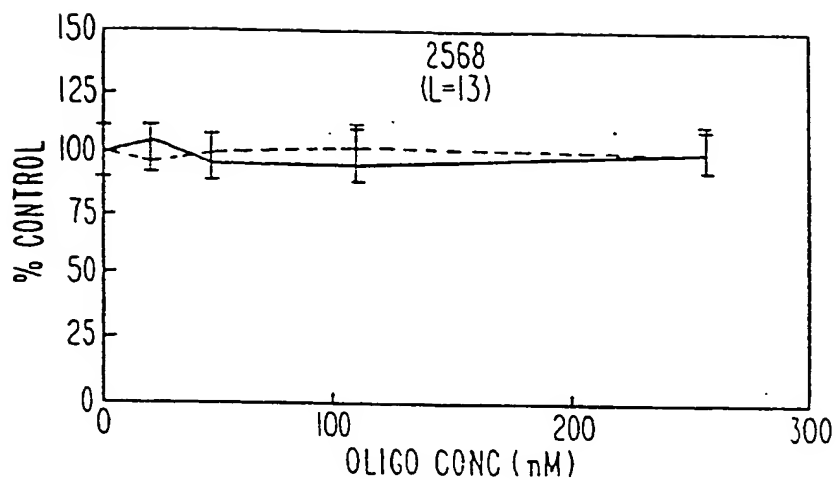
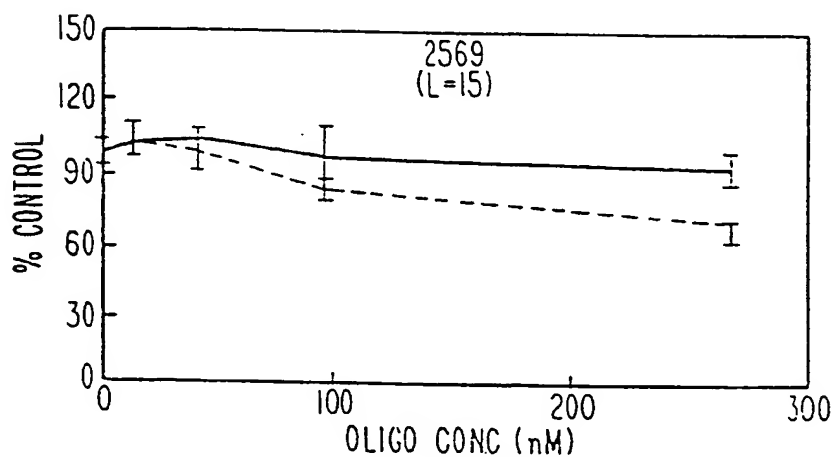
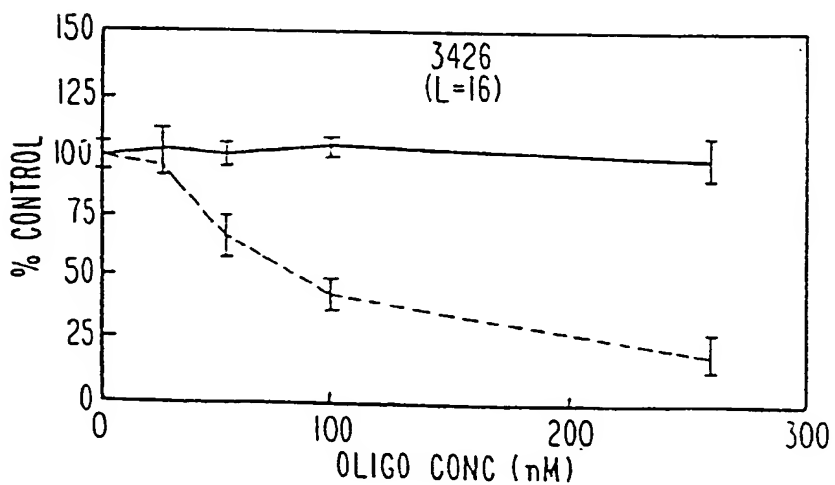
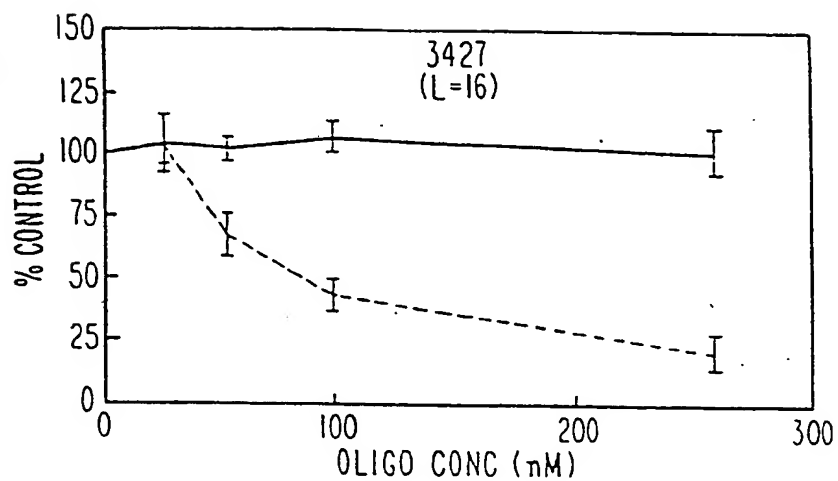
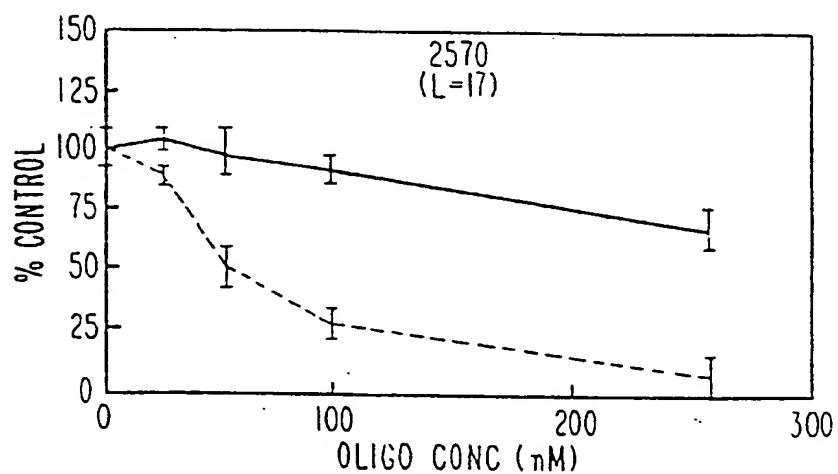
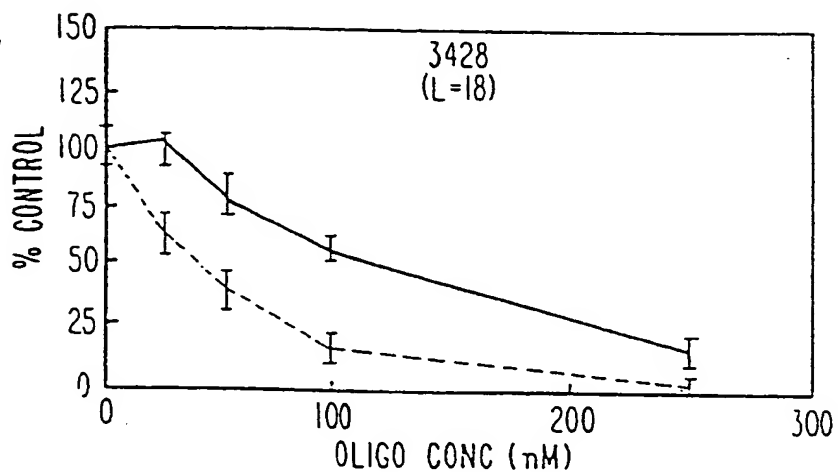
Fig. 1a***Fig. 1b******Fig. 1c***

Fig. 1dFig. 1eFig. 1f

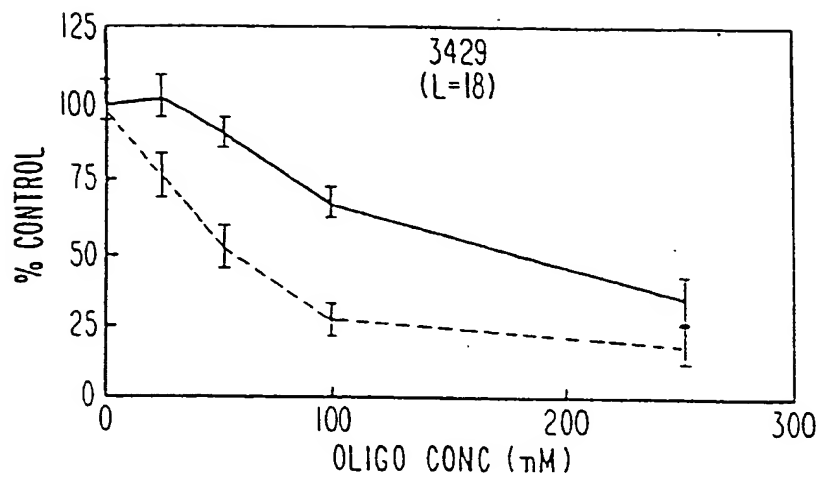


Fig. 1g

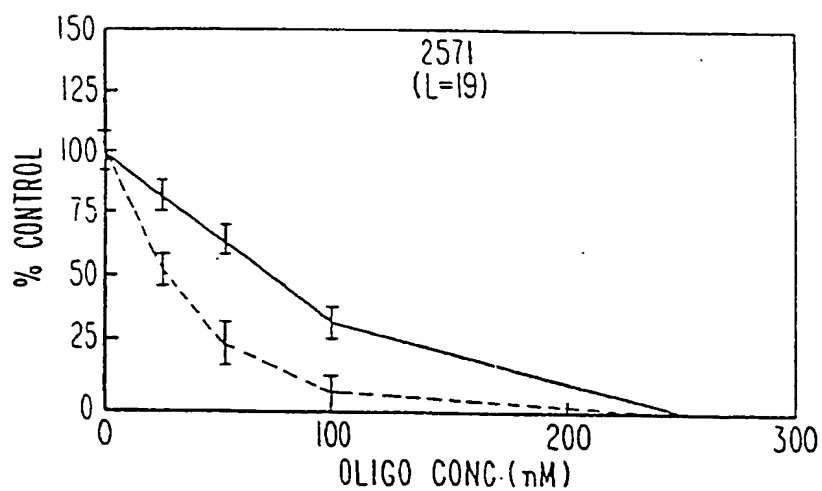


Fig. 1h

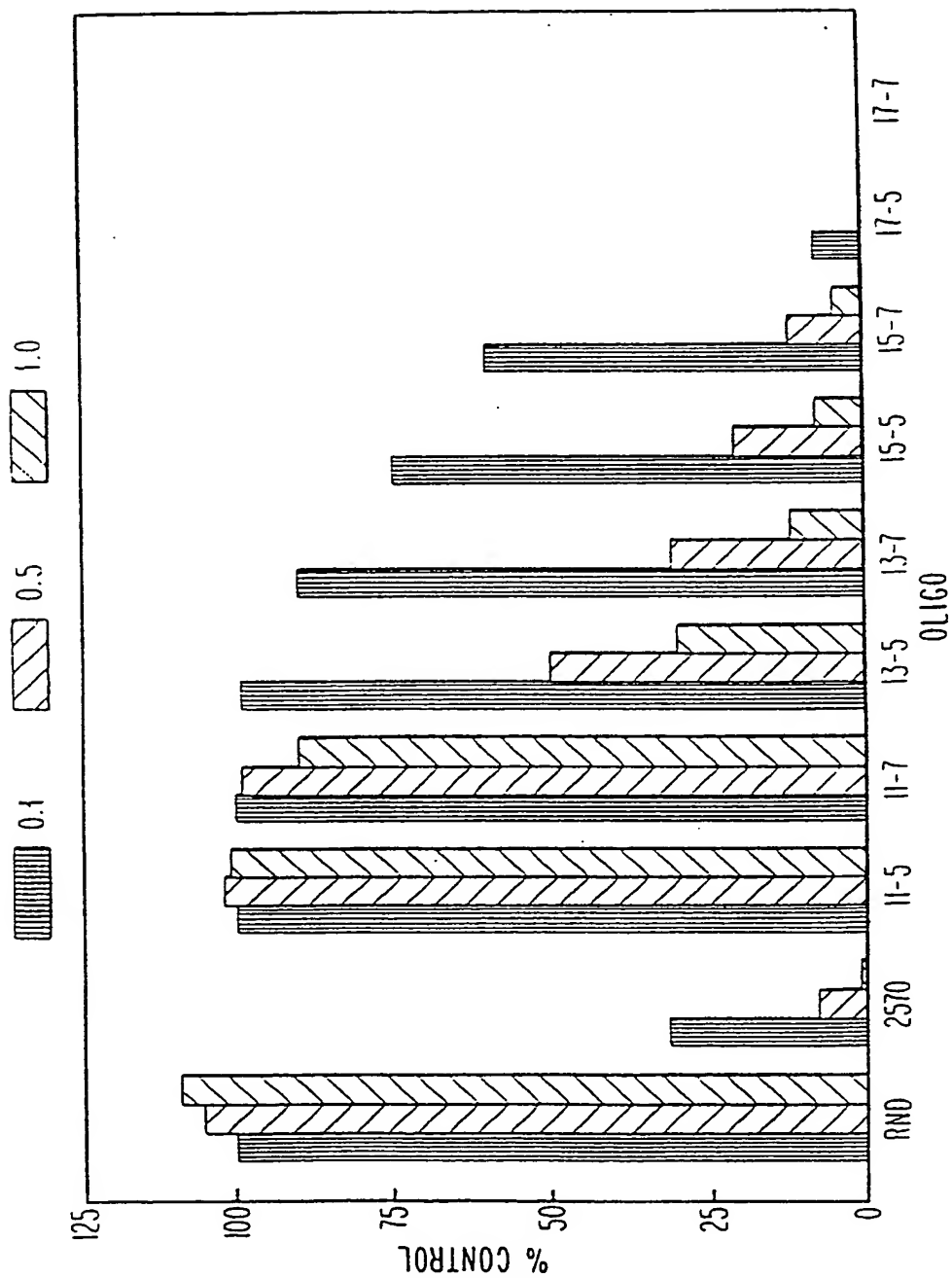
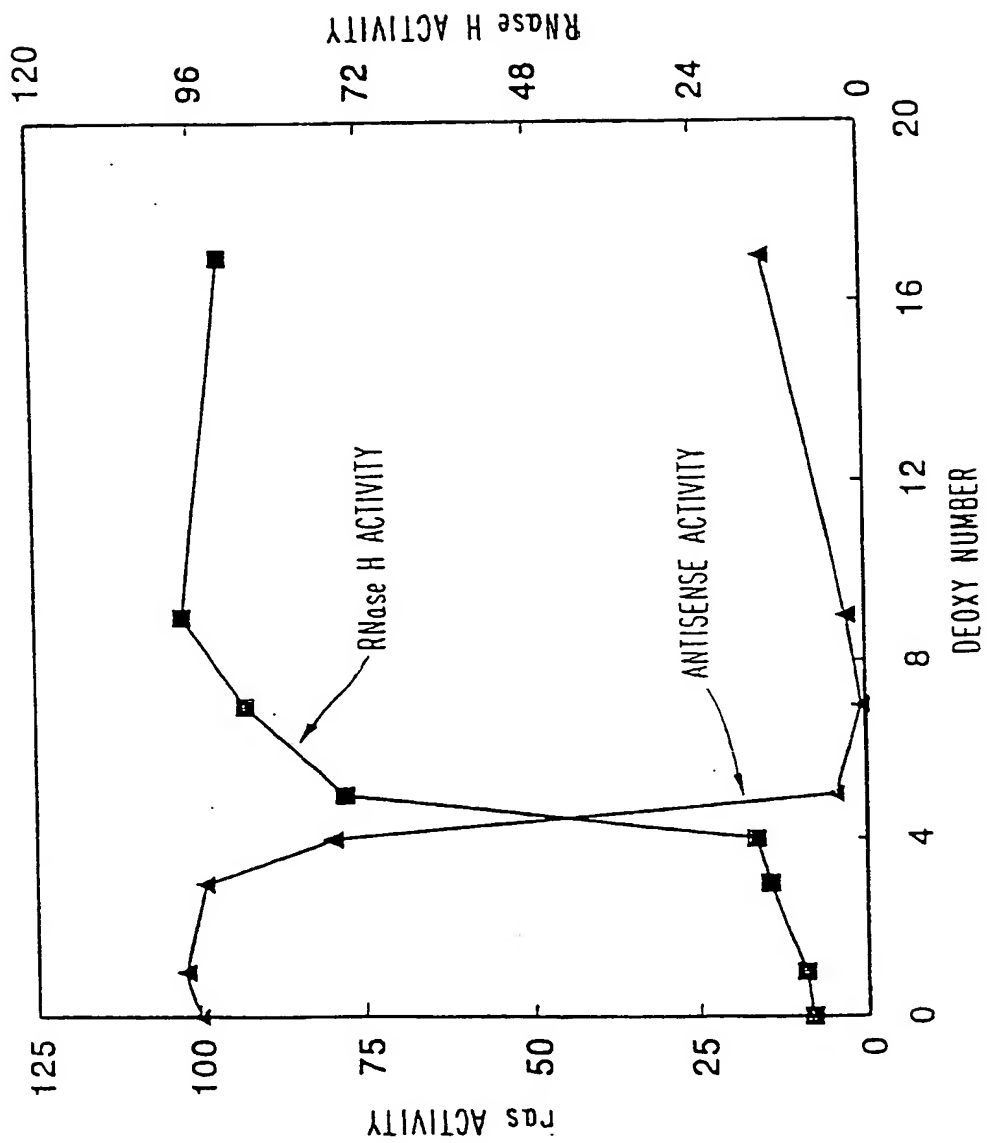
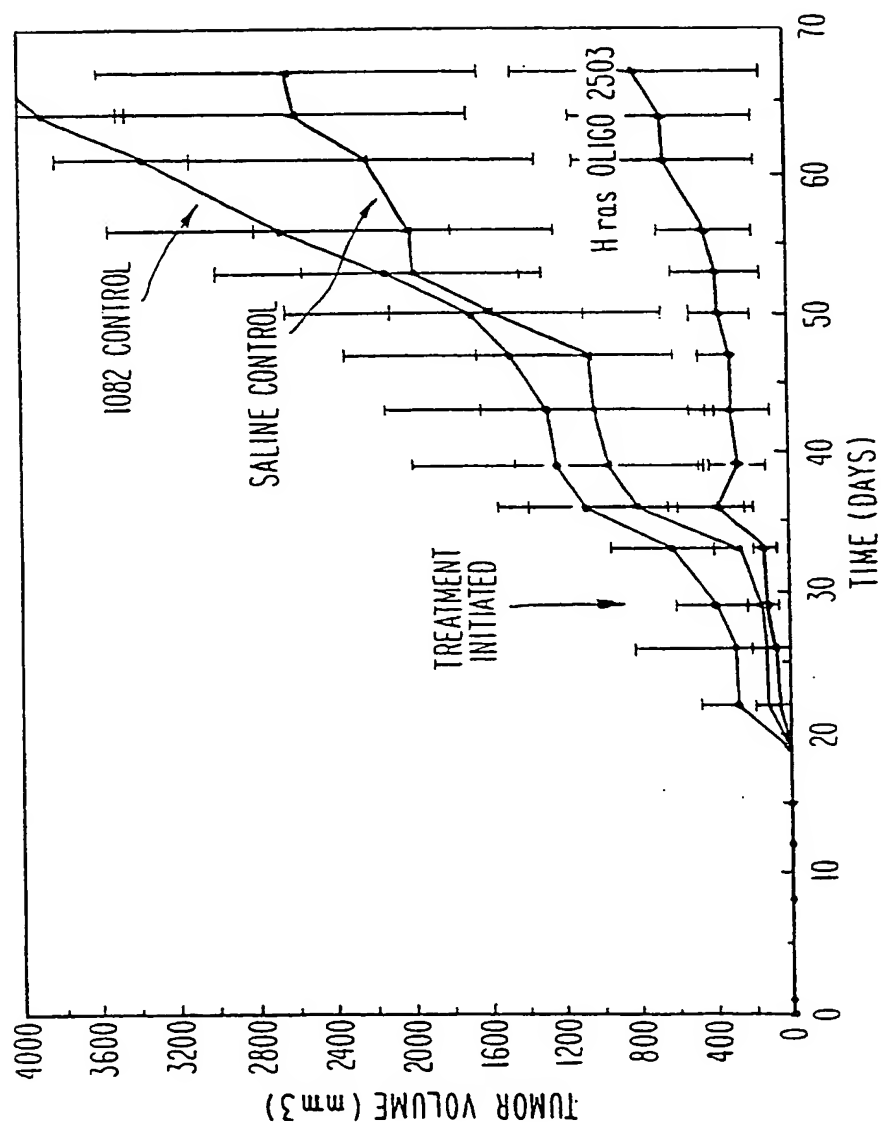
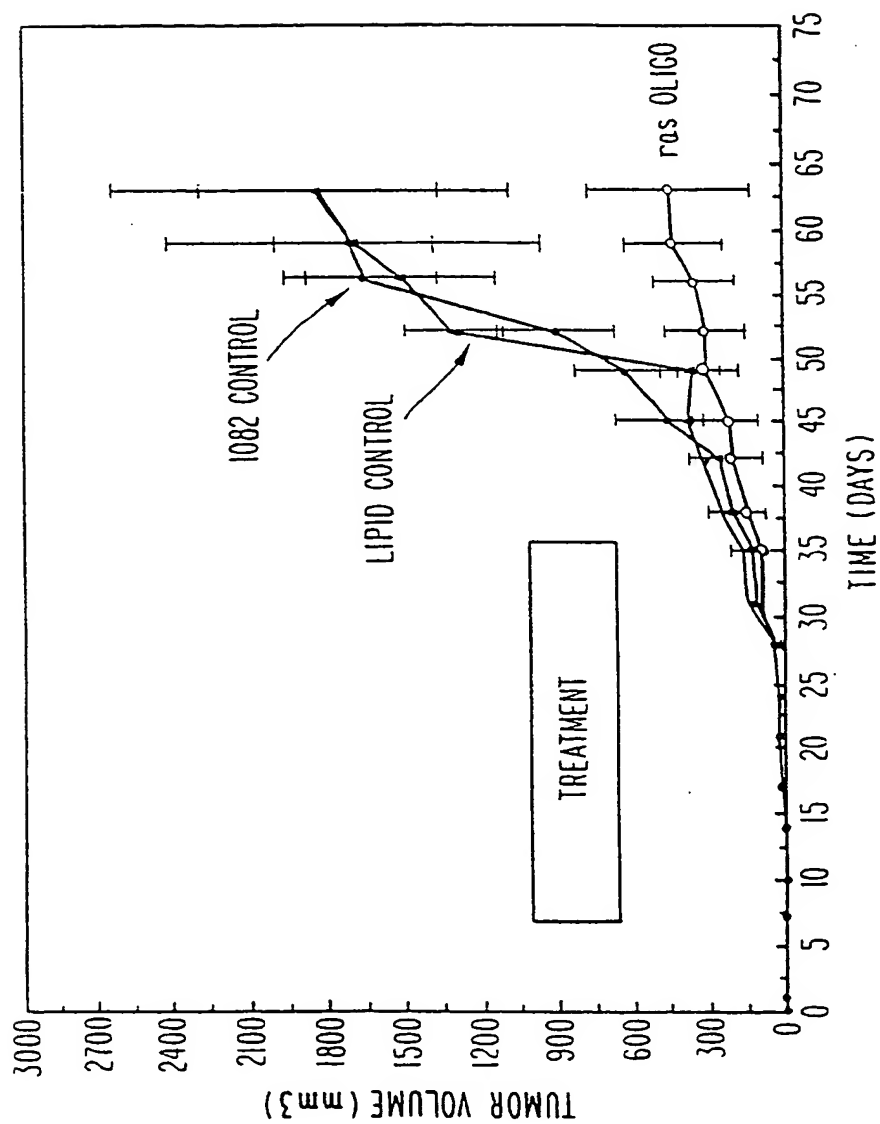
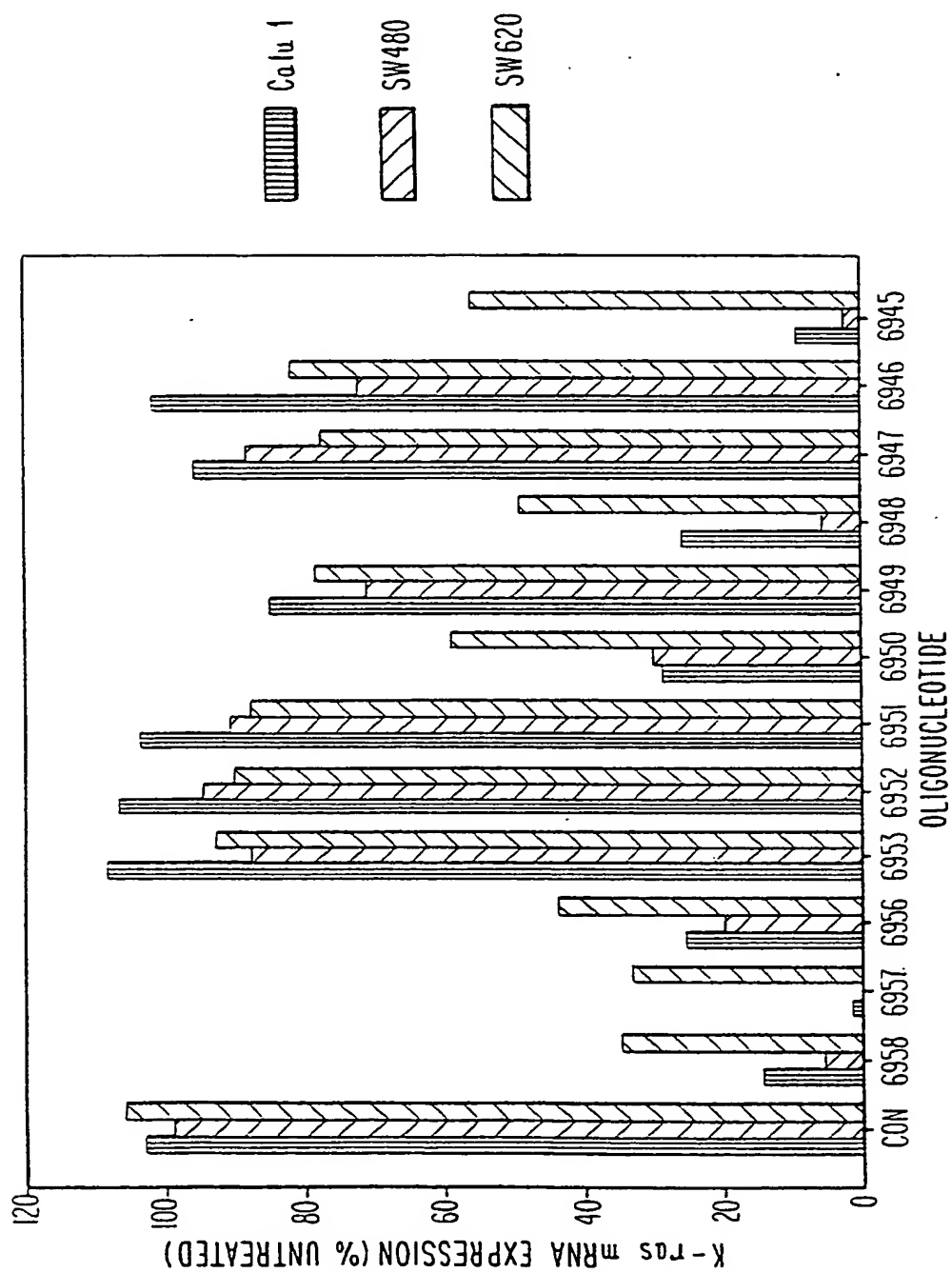


Fig. 2

*Fig. 3*

**Fig. 4**

*Fig. 5*

*Fig. 6*

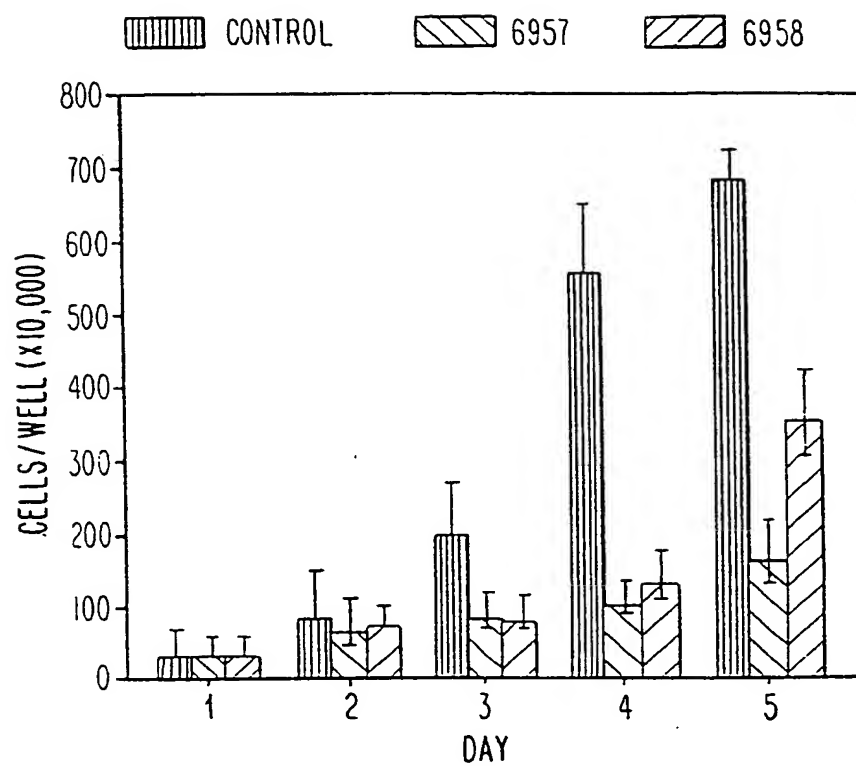
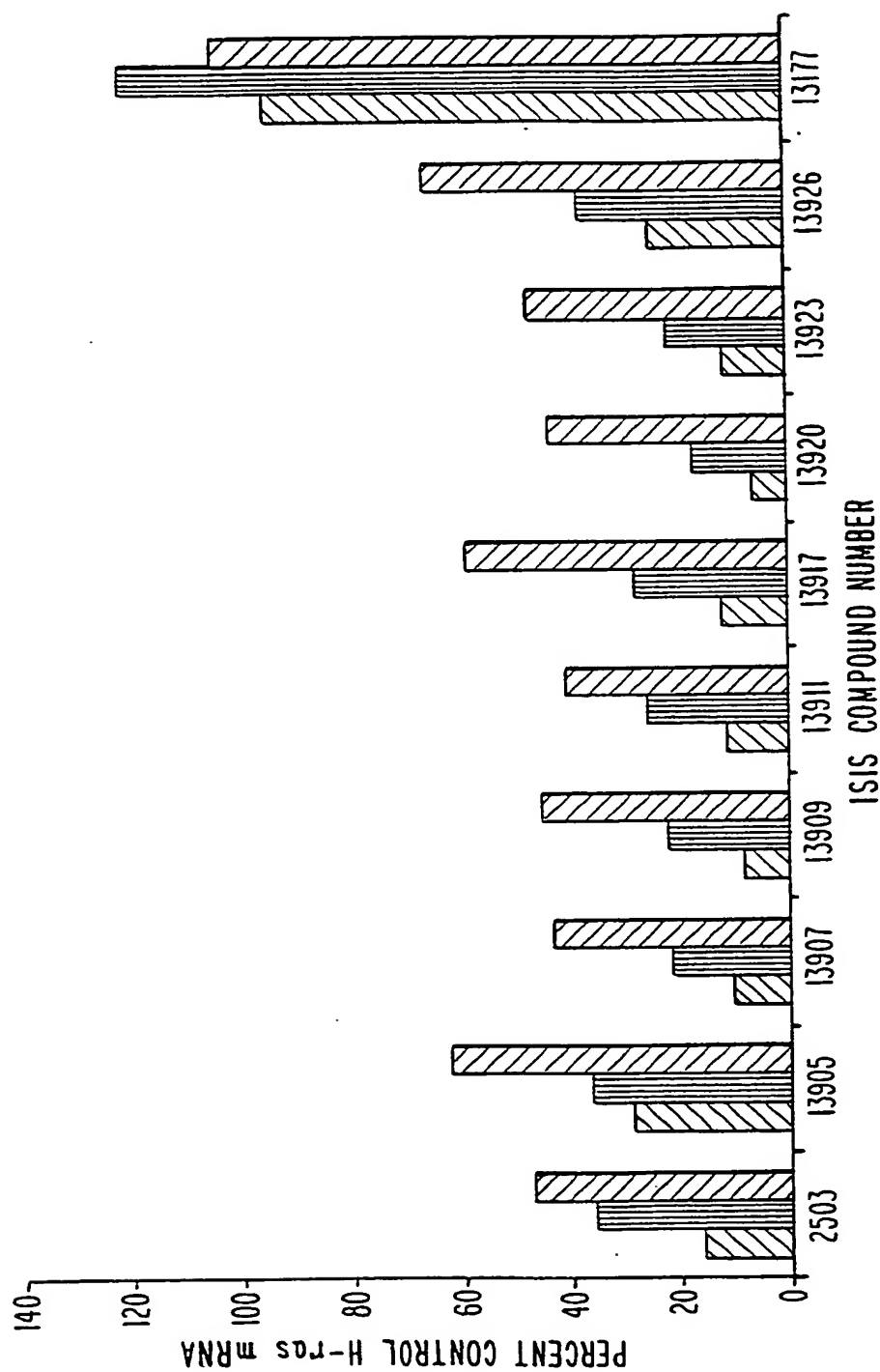


Fig. 7

***Fig. 8***

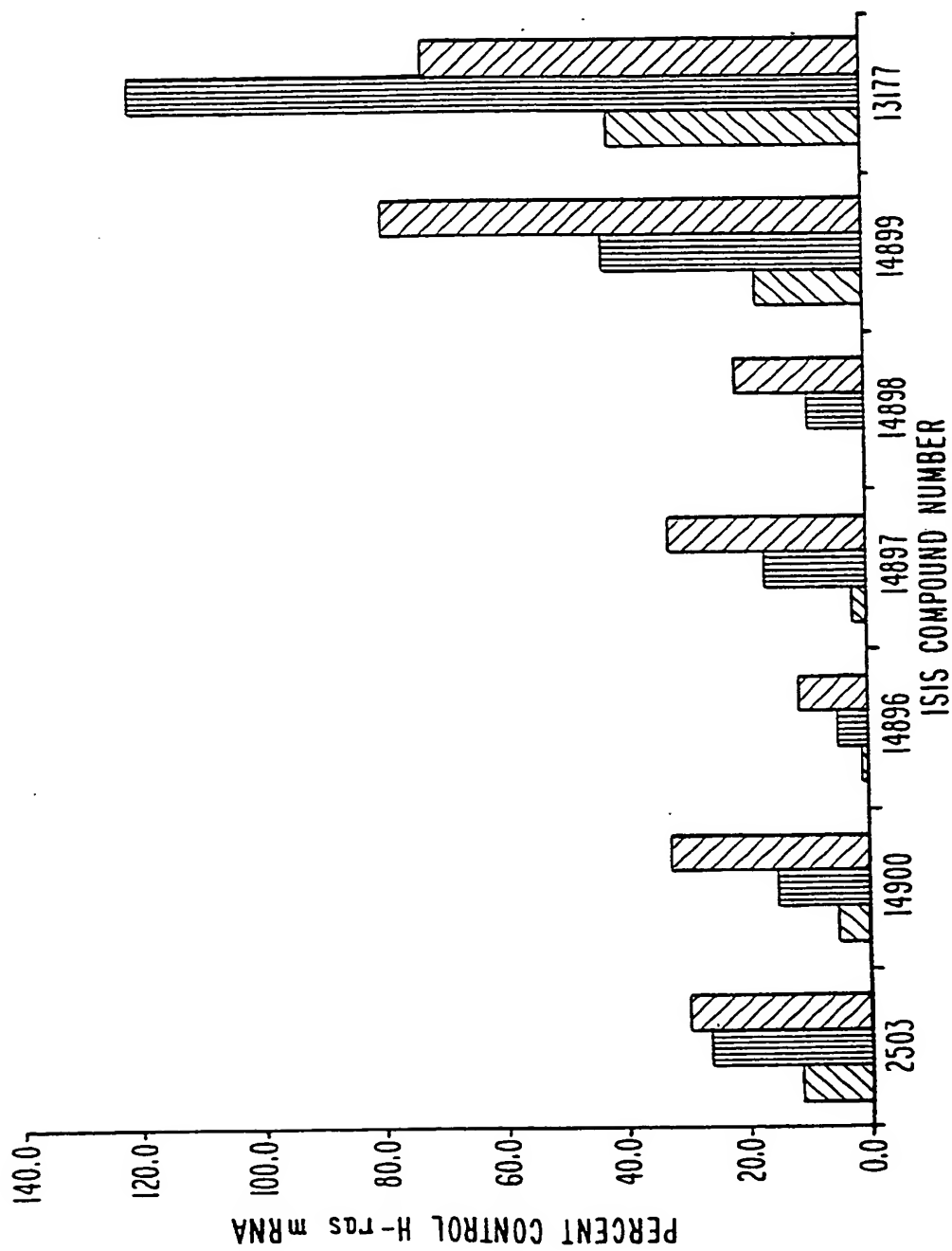
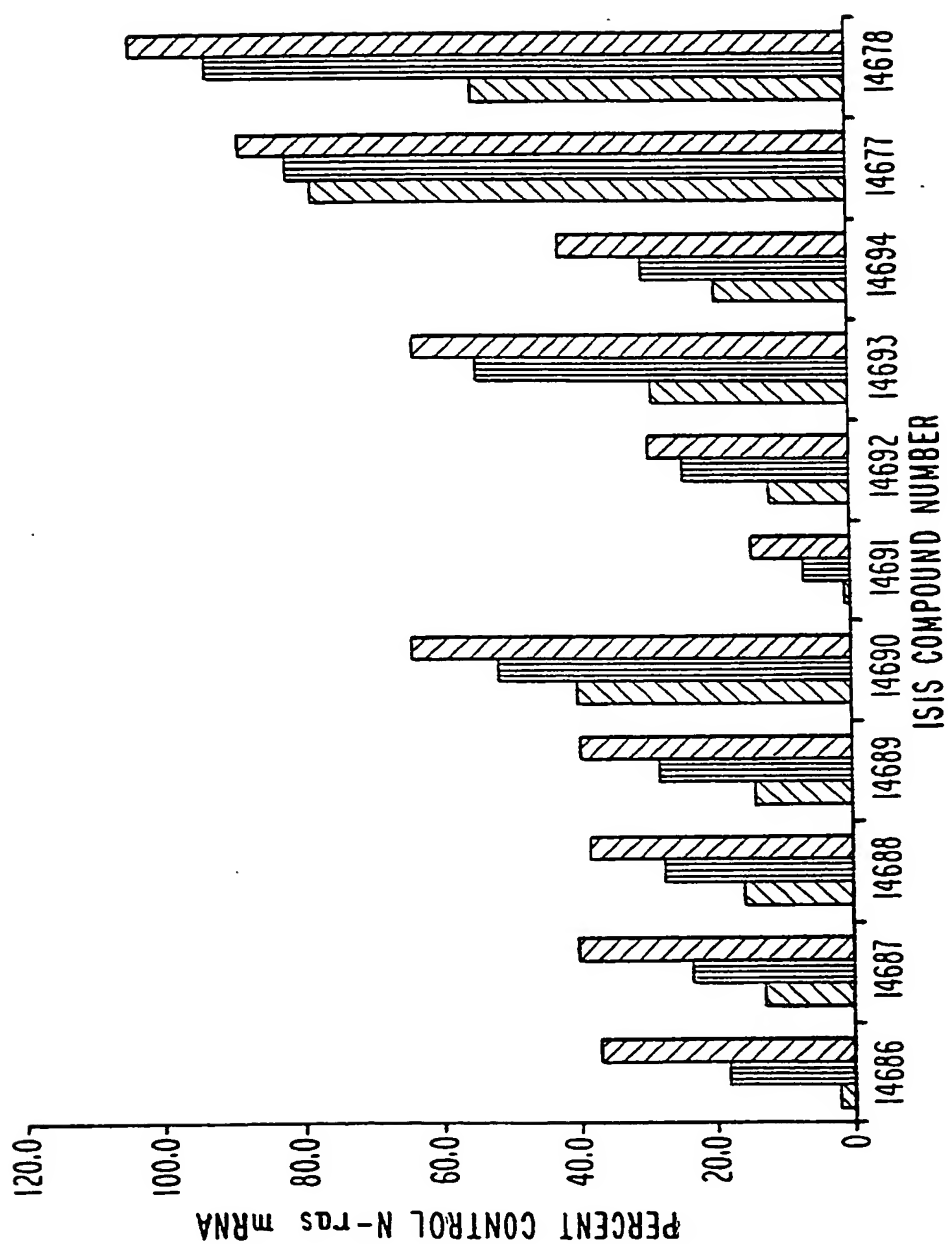


Fig. 9

**Fig. 10**

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/13966

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : C12Q 1/68, 1/70; C12P 19/34; C07H 21/02, 21/04; A61K 48/00

US CL : 435/5, 6, 91.2; 536/24.3, 24.31, 24.32, 24.33, 24.5; 514/44

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/5, 6, 91.2; 536/24.3, 24.31, 24.32, 24.33, 24.5; 514/44

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 4,871,838 A (BOS et al) 03 October 1989, see entire document.	1-9
Y	US 5,087,617 A (SMITH) 11 February 1992, see entire document.	1-9
Y	HALL et al. Human N-ras cDNA cloning and structure. Nucleic Acids Research. 1985, Vol. 13, No. 14, pages 5255-5268, see entire document.	1-9
Y	Database Medline on Dialog, US National Library of Medicine. DAAKA et al. "Target dependence of antisense oligodeoxynucleotide inhibition of c-Ha-ras p21 expression and focus formation in T24 transformed NIH3T3 cells." Oncogene Research. 1990, Vol. 5, Abstract only, see entire document.	1-9

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
A document defining the general state of the art which is not considered to be of particular relevance	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
E earlier document published on or after the international filing date	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*G* document member of the same patent family
O document referring to an oral disclosure, use, exhibition or other means	
P document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

31 AUGUST 1998

Date of mailing of the international search report

28 SEP 1998

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231

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Authorized officer

JEFFREY FREDMAN

Telephone No. (703) 308-0196

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/13966

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	SAISON-BEHMOARAS et al. Short modified antisense oligonucleotides directed against Ha-ras point mutation induce selective cleavage of the mRNA and inhibit T24 cells proliferation. EMBO J. 1991, Vol. 10, No. 5, pages 1111-1118, see entire document.	1-9
Y	UHLMANN et al. Antisense oligonucleotides: A new therapeutic principle. Chemical Reviews. June 1990, Vol. 90, No. 4, pages 543-584, see entire document.	1-9
Y	AGRAWAL et al. Site specific excision from RNA by RNase H and mixed-phosphate-backbone oligodeoxynucleotides. Proceedings of the National Academy of Sciences USA. February 1990, Vol. 87, pages 1401-1405, see entire document.	1-9
Y	INOUE et al. Sequence-dependent hydrolysis of RNA using modified oligonucleotide splints and RNase H. FEBS Letters. May 1987, Vol. 215, No. 2, pages 327-330, see entire document.	1-9

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/13966

B. FIELDS SEARCHED

Electronic data bases consulted (Name of data base and where practicable terms used):

APS, MEDLINE, BIOSIS, CAPLUS, WPIDS, CANCERLIT
search terms: ras, n, ki, oligonucleotide, DNA, RNA, nucleic, polynucleotide, antisense, inhibition, stop, block, prevent, modulate, cancer, oncogene, carcinogenesis.

